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UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCE
(Administrative Patent Judge Sally Gardner Lane)

HARUO **SUGANO**,
MASAMI MURAMATSU, and TADATSUGU TANIGUCHI

Junior Party
(Patent 5,514,567 and 5,326,859; Application 08/463,757)
v.

DAVID V. **GOEDDEL**,
and ROBERTO CREA

Senior Party
(Application 07/374,311; Patent 5,460,811).

Patent Interference Nos. 105,334 and 105,337(SGL)
(Technology Center 1600)

DECLARATION OF THOMAS M. ROBERTS, PH.D.

Sugano Exhibit 1017
Fiers v. Sugano
Interference 105,661

Sugano EXHIBIT 2016
Sugano v. Goeddel
Interference No. 105,334 and 105,337

REFERENCES CITED BY THE DECLARATION

Tab A: Curriculum Vitae

Tab B: Greenberg et al., New England J. Med., 295(10): 517-522 (1976); (Ex. 2071)

Tab C: Friedman, J. Natl. Cancer Inst., 60(6): 1191-1194 (1978); (Ex. 2072)

Tab D: Anonymous Research Disclosure, "The production of interferon by 'genetic engineering,'" Kenneth Mason Publications, Ltd., (Jul. 1979); (Ex. 2070)

Tab E: Backman and Ptashne, Cell, Vol. 13, pp. 65-71, (Jan. 1978); (Ex. 2095)

Tab F: Gilbert and Villa-Komaroff, Sci. Am. 242(4):74-94 (Apr. 1980); (Ex. 2099)

Tab G: Taniguchi et al., Proc. Jap. Acad. Ser. B, 55, 464-469 (1979); (Ex. 2095)

Tab H: Ratzkin et al., Proc. Natl. Acad. Sci. USA, 74:487-491 (Feb. 1977); (Ex. 2078)

Tab I: Vapnek et al., Proc. Natl. Acad. Sci. USA, 74:3508-3512 (Aug. 1977), (Ex. 2079)

Tab J: Itakura et al., Science, 198:1056-1063 (Dec. 1977), (Ex. 2080)

Tab K: Villa-Komaroff et al., Proc. Natl. Acad. Sci. USA, 74:3727-3731 (Aug. 1978), (Ex. 2081)

Tab L: Mercereau-Puijalon et al., Nature, 275:505-510 (Oct. 1978), (Ex. 2082)

Tab M: Chang et al., Nature, 275:617-624 (Oct. 1978), (Ex. 2083)

Tab N: Fraser and Bruce, Proc. Natl. Acad. Sci. USA, (Dec. 1978), (Ex. 2084)

Tab O: Seeburg et al., Nature, 276:795-798 (Dec. 1978), (Ex. 2085)

Tab P: Goeddel et al., Proc. Natl. Acad. Sci. USA, 76:106-110 (Jan. 1979), (Ex. 2086)

Tab Q: Schell et al., Gene, 5:291-303 (Jan. 1979), (Ex. 2087)

Tab R: Bach et al., Proc. Natl. Acad. Sci. USA, 76:386-390 (Jan. 1979), (Ex. 2088)

Tab S: Burrell et al., Nature, 279:43-47 (May 1979), (Ex. 2089)

Tab T: Martial et al., Science, 205:602-607 (Aug. 1979), (Ex. 2090)

Tab U: Goeddel et al., Nature, 281:544-548 (Oct. 1979), (Ex. 2021)

Tab V: U.S. Patent No. 5,460,811 (Ex. 2003)

Tab W: Taniguchi et al., Nature, (June 19, 1980), Vol. 285, pp. 547-549 (Ex. 2044)

Tab X: Wilson et al., Proc. Natl. Acad. Sci. USA, 76:5631-5635 (Nov. 1979), (Ex. 2092)

Tab Y: Roberts et al., Proc. Natl. Acad. Sci. USA, 76:5596-5600 (Nov. 1979), (Ex. 2093)

Tab Z: Emtage et al., Nature, 283:171-174 (Jan. 1980), (Ex. 2094)

Tab AA: Roberts et al., Proc. Natl. Acad. Sci. USA, Vol. 76, No. 2, pp. 760-764 (Feb. 1979) (Ex.2096)

Tab BB: Ptashne et al., U.S. Patent No. 4,332,892 (Ex. 2004), issued June 1, 1982, filed Jan. 10, 1980, which is a continuation of U.S. Serial No. 3,102, filed Jan. 15, 1979, now abandoned

Tab CC: Ptashne et al., U.S. Patent No. 4,418,149 (Ex. 2005)

Tab DD: Goeddel et al., U.S. Patent No. 4,342,832 (Ex. 2006)

Tab EE: Itakura and Riggs, U.K. Patent Application 2007676 (Ex. 2097)

Tab FF: Gilbert et al., European Patent Application 79301054.7 (Ex. 2098)

Tab GG: Curtiss, Ann. Rev. Microbiol., 30:507-33 (1976)(Ex. 2067)

Tab HH: Wu and Bahl, Prog. Nucleic Acid Res. Mol. Biol. 21:101-41 (1978) (Ex. 2068)

Tab II. Erlich et al., Cell, Vol. 13, pp. 681-689 (1978)(Ex. 2069)

Tab JJ: Goeddel Clean Copy of Claims Filed in Interference No. 105,334

Tab KK: Goeddel Clean Copy of Claims Filed in Interference No. 105,337

Tab LL: Taniguchi et al., Gene, 10:11-15 (May 19, 1980) (Ex. 2060)

Tab MM: Preprint Manuscript by Taniguchi et al. entitled "Human leukocyte and fibroblast interferons are structurally related" (Exhibit 2023)

Tab NN: English Translation of Japanese Patent Application No. 139289/79 filed on October 30, 1979 (Ex. 2015)

Tab OO: Knight et al., Science, 207:525-526 (Feb. 1980) (Ex. 2066)

Tab PP: Fujisawa et al., J. Biol. Chem., 253(24): 8677-8679 (1978) (Ex. 2077)

Tab QQ: Guarente et al., Cell, 20:543-553 (June 1980)(Ex. 2046)

Tab RR: Goeddel U.S. Application Serial No. 07/374,311 (Ex. 2007)

Tab SS: Knight and Hunkapiller, "Purification, Amino Acid Composition, and N-Terminal Amino Acid Sequence of Human Fibroblast Interferon," from Annals of the New York Academy of Sciences, Vol. 350, "Regulatory Functions of Interferons," Eds. Vilcek et al., (1980) (Ex. 2053)

Tab TT: Backman, K. et al., Proc. Natl. Acad. Sci. USA, Vol. 73, No. 11, pp. 4174-4178 (1976) (Ex. 2108)

Tab UU: Goeddel U.S. Patent No. 5,460,811 (Ex. 2003)

Tab VV: Ed. Szuromi, This Week in Science, "Glycosylation in Bacteria," Science, 298:1677 (2002) (Ex. 2073)

Tab WW: Havell et al., Virology, 63: 475-483 (1975) (Ex. 2074)

Tab XX: Bose et al., J. Biol. Chem., 251(6): 1659-1662 (1976) (Ex. 2075)

Tab YY: Havell et al., J. Biol. Chem., 252(12): 4425-4427 (1977) (Ex. 2076)

Tab ZZ: Taniguchi et al., Proc. Natl. Acad. Sci. USA, Vol. 77, No. 9, pp. 5230-5233 (Sept. 1980) (Ex. 2122)

I, THOMAS M. ROBERTS, declare and state as follows:

Bibliographic Information:

1. I reside at 13 Berkeley Street, Cambridge, MA 02138.

2. I received a Ph.D. degree in Biochemistry and Molecular Biology from Harvard University in 1976.

3. I was a post-doctoral fellow in the laboratory of Dr. Mark Ptashne at Harvard University between 1976 and December 1980.

4. I presently hold the position of Professor of Pathology at Harvard Medical School. I am also the Chairman of the Division of Medical Sciences at Harvard University, the Faculty Dean at Harvard Medical School, and the Co-Chair of the Department of Cancer Biology at the Dana Farber Cancer Institute.

5. My complete academic background, professional experience and honors are set forth in my curriculum vitae, a copy of which is attached hereto as Tab A.

The Problem of Mass-Producing Interferon -- The Solution of Bacterial Protein Expression

6. Human fibroblast and leukocyte interferons were the subject of intense interest well before March 19, 1980. Numerous groups were studying the interferons because at the time, the anti-viral ability of interferons was well-known and groups were also actively investigating potential anti-cancer applications. For example, the 1976 New England Journal of Medicine publication by Greenberg et al. (Tab B) reported the effect of human leukocyte interferon on Hepatitis B Virus infection in human patients; the 1978 Journal of the National Cancer Institute publication by Friedman (Tab C) reviewed the antiviral activity and anti-tumor effects of interferons.

7. Corporations and laboratories were interested in expressing interferon in bacteria because it was well-known in 1979 that interferon clinical research was hampered by the problem of producing enough interferon for wide-scale clinical studies. Corporations and laboratories involved in the cloning and/or expression of human fibroblast or leukocyte interferon in early 1980 included at least the Ptashne Laboratory, Genentech, Biogen, the Weissmann Laboratory, and the Fiers Laboratory. The problem of interferon mass production was published in the July 1979 Anonymous Research Disclosure entitled "The production of interferon by 'genetic engineering,'" (Tab D):

> Human interferon is usually produced from either leukocytes or from fibroblast cells. Up to now, clinical studies have employed human leukocyte interferon. One cell is able to make only minute quantities of interferon so large quantities of human cells are needed for production. The problem of shortage of human interferon for clinical studies is still the major drawback. It is for this reason that some people have turned their attention to a line of transformed human lymphblastoid cells. These cells can be grown in very large numbers and hence larger quantities of interferon can be produced. However, probably the best way to produce large quantities of interferon will be to introduce the gene(s) for interferon production into bacteria. This technique has already been demonstrated with other mammalian genes eg somatostatin, insulin, ovalbumin, dihydrofolate reductase and hepatitis antigen.

(See Tab D at first page, 2nd ¶.)

8. Other publications at the time explicitly suggest the use of bacteria to express eukaryotic proteins. For example, the Backman and Ptashne January 1978 publication entitled "Maximizing Gene Expression on a Plasmid Using Recombination in Vitro," (Tab E) states:

> Experiments reported in this paper utilize recombination in vitro to place one or more copies of a strong promoter, the lac promoter, at varying distances from the cI (repressor) gene of bacteriophage λ . . . . In our most potent fusion, we created a "hybrid" ribosome binding site; the *lac* promoter and adjacent sequences were positioned so that the *lac Z* gene SD sequence was 8 base pairs upstream from the ATG of the *cI* gene. In principle, this strategy should elicit high levels of expression in E. coli of any gene, whatever its source.

(See Tab E at p. 65, lt. col., last ¶ to rt. col., 1st ¶.) Another example is the Gilbert and Villa-Komaroff April 1980 article "Useful Proteins from Recombinant Bacteria" (Tab F; Ex. 2099), which even provides the explicit suggestion to produce interferon: "Bacteria into which nonbacterial genes have been introduced are able to manufacture nonbacterial proteins. Among the proteins made by recombinant-DNA methods are insulin and interferon." (See Tab F at Abstract.) In fact, in a 1979 publication, Taniguchi himself expresses the idea to mass produce human fibroblast interferon: "In conclusion, we have reportred [sic] in this paper the construction of the recombinant plasmid DNA which contains human fibroblast interferon gene sequence. ... The recombinant plasmid we have constructed will be useful not only for defining the hitherto unknown structure of the interferon gene but all for the attempts of the mass production of this protein." (See Tab G [Taniguchi et al., Proc. Japan. Acad., 55, Ser. B (1979)] at p. 468, 2nd ¶.)

9. Thus, it is my opinion that because of the problem of interferon mass-production in mammalian cells, the logical solution at the time was to produce interferon in bacteria. As mentioned in the Anonymous Research Disclosure article (Tab D), research into mammalian protein production in bacteria was well underway (see Scope and Content of the Prior Art sections below), and considering the overwhelming interest in interferon, an attempt to express interferons in bacteria was an obvious strategy.

Scope and Content of the Prior Art: Examples of Protein Expression in Bacteria as of March 19, 1980

10. I have reviewed the following seventeen papers that provide some specific examples of the expression of recombinant proteins in bacteria as of March 19, 1980:

Tab H: Ratzkin et al., Proc. Natl. Acad. Sci. USA, 74:487-491 (Feb. 1977);

Tab I: Vapnek et al., Proc. Natl. Acad. Sci. USA, 74:3508-3512 (Aug. 1977);

Tab J: Itakura et al., Science, 198:1056-1063 (Dec. 1977);

Tab K: Villa-Komaroff et al., Proc. Natl. Acad. Sci. USA, 74:3727-3731 (Aug. 1978);

Tab L: Mercereau-Puijalon et al., Nature, 275:505-510 (Oct. 1978);

Tab M: Chang et al., Nature, 275:617-624 (Oct. 1978);

Tab N: Fraser and Bruce, Proc. Natl. Acad. Sci. USA, (Dec. 1978);

Tab O: Seeburg et al., Nature, 276:795-798 (Dec. 1978);

Tab P: Goeddel et al., Proc. Natl. Acad. Sci. USA, 76:106-110 (Jan. 1979);

Tab Q: Schell et al., Gene, 5:291-303 (Jan. 1979);

Tab R: Bach et al., Proc. Natl. Acad. Sci. USA, 76:386-390 (Jan. 1979);

Tab S: Burrell et al., Nature, 279:43-47 (May 1979);

Tab T: Martial et al., Science, 205:602-607 (Aug. 1979);

Tab U: Goeddel et al., Nature, 281:544-548 (Oct. 1979);

Tab X: Wilson et al., Proc. Natl. Acad. Sci. USA, 76:5631-5635 (Nov. 1979);

Tab Y: Roberts et al., Proc. Natl. Acad. Sci. USA, 76:5596-5600 (Nov. 1979); and

Tab Z: Emtage et al., Nature, 283:171-174 (Jan. 1980).

11. The February 1977 Ratzkin article (Tab H), entitled "Functional expression of cloned yeast DNA in *Escherichia coli*," raises the "key question invoked by the recent discovery of methods to biochemically construct recombinant DNAs concerns the ability of fragments of eukaryotic DNA inserted into plasmid or phage vectors to be expressed meaningfully in a bacterium such as *Escherichia coli*." (Tab H at p. 487, lt. col., 2nd ¶.) In particular, the paper raises the concerns of differences between eukaryotes and prokaryotes with respect to "RNA polymerases, ribosomal subunits, translational initiation systems, and post-transcriptional and post-translational modifications." (Id.) But because their experiments show that various yeast

genes were able to complement bacterial mutations, they conclude that "many yeast DNA segments in E. coli will be readily expressed; the barriers to translation and transcription are somehow overcome." The authors further hypothesize that "it seems possible that productive expression of yeast (and perhaps other eukaryotic) DNA in E. coli can occur more readily than has previously been anticipated." (Id. at p. 491, rt. col., 1st ¶.) Thus, in my opinion, the strategy to express eukaryotic gene loci in bacteria was present in the state of the art as of at least February 1977.

12. In the August 1977 Vapnek article (Tab I), the authors expressed the structural gene for catabolic dehydroquinase of *Neurospora crassa* (a eukaryote) in *E. coli*. The authors state that the "results presented in this communication demonstrate unequivocally that eukaryotic DNA can be faithfully transcribed and translated in a prokaryotic host." (Tab I at p. 3511, rt. col., 2nd full ¶.) The authors teach that the quinic acid gene *qa-2*$^{+}$ gene "when carried on a recombinant plasmid" "could be expressed by one of three alternative mechanisms: (i) transcription initiation from a pBR322 promoter; (ii) transcription initiation from a normal *N. crassa* promoter by a mechanism that does not require the presence of a regulatory protein; (iii) transcription initiation by the normal *N. crassa* mechanism which requires the presence of an expressed *qa-1*^{C} gene." (Id.) Thus, in my opinion, the Vapnek article provides the explicit teaching that control of transcription of a eukaryotic gene in bacteria can take place by a bacterial promoter, i.e., pBR322 promoter.

13. In the December 1977 Itakura paper, the authors express a chemically synthesized gene for the mammalian hormone somatostatin in bacteria. The gene for somatostatin, made by chemical DNA oligonucleotide synthesis, was fused into the E. coli β-galactosidase gene on the plasmid pBR322 (see Tab J at Figure 1). Thus, the somatostatin protein was expressed as a

fusion protein with β-galactosidase under the transcriptional and translational control of bacterial *lac* operon control elements. As the authors state, the "results represent the first success in achieving expression (that is, transcription into RNA and translation of that RNA into a protein of a designed amino acid sequence) of a gene of chemically synthesized origin." (See Tab J at p. 1062, 3rd col., 2nd ¶.) Thus, in my opinion, the Itakura article provides the explicit teaching that a mammalian gene can be transcribed and translated in bacteria via bacterial, i.e., *lac* operon, control elements.

14. In the August 1978 Villa-Komaroff paper (Tab K), the authors express insulin by inserting a rat preproinsulin cDNA into the bacterial penicillinase gene such that a penicillinase-insulin fusion protein is produced. The underlying question for the paper was: "Can the structural information for the production of a higher cell protein be inserted into a plasmid in such a way as to be expressed in a transformed bacterium." (Tab K at p. 3727, lt. col., 2nd ¶.) The authors were interested in whether a mammalian protein's structure could be reproduced by the bacterial machinery, which was answered in the affirmative, "[n]ot only is the fused cDNA sequence expressed as a chain of amino acids, but also the polypeptide folds so as to reveal insulin antigenic shapes." (Tab K at p. 3730, lt. col., 3rd full ¶.) Thus, in my opinion, this Villa-Komaroff paper shows that a mammalian protein can be produced by bacteria.

15. In the October 1978 Chang article (Tab M), the authors describe the expression of a functional mouse dihydrofolate reductase (DHFR) enzyme in bacteria. The authors first comment upon the progress of recombinant protein expression in bacteria at the time, "[v]ery recently, a protein containing amino acids of rat proinsulin was shown to be made by bacteria ... [i]t is not known, however, whether the mammalian peptide components of such immunologically reactive hybrid proteins have functional biological activity." (Tab M at p. 617,

rt. col., 1st ¶.) Chang and colleagues express DHFR as a fusion with the bacterial β-lactamase protein and test the expressed proteins for functional biological activity. The authors conclude "[t]he findings reported here indicate that the bacterial clones we have constructed are synthesising and phenotypically expressing DHFR encoded by mouse cDNA sequences ..." (Id. at p. 622, rt. col., 2nd ¶.) Thus, in my opinion, the Chang article advanced the state of the art to show that a mammalian protein produced in bacteria can maintain functionality.

16. In the October 1978 Mercereau-Puijalon publication (Tab L), the authors provide another example of a mammalian protein produced in bacteria as a fusion protein. In this paper, the chick ovalbumin gene was inserted at the beginning of the E. coli *lacZ* gene, which codes for β-galactosidase, such that a fusion protein of "eight amino acids of β-galactosidase and 381 amino acids of ovalbumin" was produced. (Tab L at p. 509, rt. col., 1st ¶). Not having the benefit of the Chang paper described immediately above, the authors conclude that their studies are "the first example of synthesis by *E. coli* cells, of a long polypeptide chain from a higher eukaryote." (Id. at p. 510, lt. col., 1st full ¶). The authors also conclude, "[t]here is no theoretical reason, in our opinion, that the site of the junction between the *lac* and the ovalbumin sequences could not be moved down to the ATG initiator codon, leading to the synthesis of an accurate product" and "our experiments demonstrate the feasability of engineering bacteria for a potentially very large number of uses." (Id.) In my opinion, this paper provides yet another example where a very large mammalian protein (381 amino acids) could be expressed in bacteria.

17. The December 1978 Fraser publication (Tab N) discloses the production of a full-length chicken ovalbumin protein. The authors fused the full-length ovalbumin cDNA downstream of the *lac* control region such that the ovalbumin was presumably produced as a

fusion with 18 amino acids of β-galactosidase. "From Fig. 5, showing the first 107 bases of this mRNA, it can be seen that if protein synthesis is initiated at the β-galactosidase AUG (position 39) the ovalbumin molecule will be synthesized with 18 extra amino acids on its amino terminus." (Tab N at p. 5939, lt. col., 2nd ¶). The authors conclude, "the microbial production of significant amounts of chicken ovalbumin reported here demonstrates that the E. coli cellular machinery may be utilized to synthesize a eukaryotic protein that is stable in the bacterial intracellular environment. There is no reason to believe that the synthesis of other high molecular weight animal proteins will be any more difficult than that of ovalbumin." (Id. at p. 5940, lt. col., 5th ¶). In my opinion, the Fraser publication builds upon the growing successes of mammalian fusion protein expression in bacteria.

18. In the December 1978 publication by Seeburg et al. (Tab O), the authors produce a β-lactamase-rat pre-growth hormone fusion protein, "a chimaeric protein of 395 amino acids ... containing the N-terminal 181 amino acids of the β-lactamase precursor covalently linked to 214 residues of rat pre-GH." (Tab O at p. 797, lt. col. 1st ¶.) The authors conclude: "Our findings and those of Villa-Komaroff et al. [Tab K] indicate that coding sequences from higher organisms can be expressed in bacteria. Thus, it should be possible to produce biologically important peptides with the use of recombinant DNA techniques and naturally occurring structural gene sequences." (Tab O at p. 798, rt. col. 3rd ¶.) Thus, as stated, the prior art continues to build a strong motivation to express mammalian proteins in bacteria.

19. In the January 1979 Goeddel paper (Tab P), β-galactosidase-human insulin A chain and β-galactosidase-human insulin B chain fusion proteins were produced in bacteria. After production of these fusion proteins, the insulin peptides were cleaved from β-galactosidase and purified. The authors summarize their findings as follows: "(i) Radioimmune activity has

been detected for both chains. (ii) The DNA sequences obtained after cloning and plasmid construction have been directly verified to be correct as designed. Because radioimmune activity is obtained, translation must be in phase. Therefore, the genetic code dictates that peptides with the sequences of human insulin are being produced. (iii) The E. coli products, after cyanogen bromide cleavage, behave as insulin chains in three different chromatographic systems ..." (Tab P at p. 110, rt. col., 2nd ¶.) In my opinion, the radiommune assays show that human insulin polypeptides might have had a proper structural conformation when expressed in bacteria.

20. The January 1979 Schell (Tab Q) and Bach (Tab R) papers provide two additional examples of yeast genes expressed in bacteria.

21. The May 1979 Burrell paper (Tab S) discloses that insertion of Hepatitis B virus coding sequences into E. coli coding sequences allows for the production of bacterial-viral fusion proteins.

22. The August 1979 Martial paper (Tab T) provides yet another successful expression of a eukaryotic-bacteria fusion protein. "The human growth hormone gene sequences have been linked in phase to a fragment of the trp D gene of Escherichia coli in a plasmid vehicle, and a fusion protein is synthesized at high level (approximately 3 percent of bacterial protein) under the control of the regulatory region of the trp operon." (Tab T at p. 602, Abstract.)

23. The Oct. 1979 Goeddel paper (Tab U) provides for the "direct expression" in E. coli of human growth hormone. By "direct expression," the authors appear to mean that the mature human growth hormone (as opposed to expression of a precursor as a fusion protein) was expressed in bacteria. "This is the first time that a human polypeptide has been directly

expressed in *E. coli* in a non-precursor form." (Tab U at p. 548, rt. col., 4th ¶.) The authors accomplished this by the following plan:

> Treatment of double stranded (ds) HGH cDNA with *Hae*III gives a DNA fragment of 551 base pairs which includes coding sequences for amino acids 24-191 of HGH. We planned to clone this cDNA fragment and to clone separately a chemically synthesised DNA 'adaptor' fragment containing an ATG initiation codon and coding sequences for residues 1-23 of HGH. These two DNA fragments would be combined to form a synthetic-natural 'hybrid' gene. When inserted into a plasmid downstream from a suitable bacterial promoter and ribosome binding site, this gene could be expected to direct synthesis of fMet-HGH. The fact that most bacterial proteins do not contain N-terminal methionine residues suggests that fMet shold be efficiently removed, resulting in direct expression of HGH.

(Id. at p. 544, lt. col., 3rd ¶.) The authors state that their methods "are generally applicable to other polypeptides which are synthesized initially as inactive precursors and later processed, or for which full length cDNA transcripts are unavailable." (Id. at p. 548, rt. col., 4th ¶.) In my opinion, this paper shows that a mammalian protein can be transcribed and translated under the control of bacterial control elements, such that expression can be conducted without the need to make a bacterial-eukaryotic fusion protein.

24. In Wilson et al. (Nov. 1979) (Tab X), the authors were able to express human γ and β globins in E. coli by inserting these globin genes into bacterial plasmids.

25. My November 1979 paper ("Roberts November 1979 paper"; Tab Y) shows the expression of simian virus 40 t antigen in bacteria by making *lac* control element-cDNA gene fusions. In other words, expression was not contingent upon making a fusion to a bacterial *protein*:

> Plasmids are constructed by using recombination *in vitro* according to Roberts, T.M., Kacick, R. & Ptashne, M. (1979) *Proc. Natl. Acad. Sci. USA* 76, 760-764 in which the t antigen gene of simian virus 40 is fused to a promoter of the *Escherichia coli lac* operon. In the fusions, transcription commences at the *lac* promoter, and, in some of the fusions, translation begins at the ATG initiator codon of the t gene. This translation is directed most efficiently by those

> plasmids in which the *lac* sequences abut the t gene such that a hybrid ribosome binding site is encoded. In this case, the Shine-Delgarno sequence is of *lac* origin but the ATG derives from the *t* gene. [....] We conclude that these bacteria are producing a protein, the sequence of which is identical to that of authentic t antigen unfused to other polypeptides.

(Tab Y at p. 5596 at Abstract.) This paper departs from the prior art discussed immediately hereinabove by: "Most of the reports of synthesis of higher eukaryotic proteins in bacteria have described the production of fusion proteins composed of both prokaryotic and eukaryotic sequences." (Id. at p. 5600, lt. col., 5th ¶.) But this paper teaches that a eukaryotic gene (a eukaryotic gene because the viral gene is expressed in eukaryotes), beginning at its ATG, can be expressed in bacteria when the gene is fused to bacterial control sequences: "Our experiments show that a message bearing a hybrid ribosome-binding site - i.e., sequences derived partly from the bacterium (the SD [Shine-Delgarno] sequence) and partly from the eukaryotic gene (the ATG, etc.) - can be correctly translated into protein. This provides a rational approach to the problem of obtaining expression of eukaryotic genes in bacteria." (Id.) Thus, in my opinion, the prior art as of November 1979 provides at least two specific examples (this paper and the Goeddel 1979 paper discussed in paragraph 23 above) of producing mammalian proteins in bacteria by directly expressing the mammalian gene (i.e., from the gene's ATG and not from a bacterial gene's ATG) under the control of a bacterial promoter and a ribosomal binding site.

26. The Jan. 1980 Emtage paper (Tab Z) provides another example as of March 19, 1980 that a eukaryotic gene can be expressed from its own ATG. The paper states:

> We have previously cloned at the HindIII site of pBR322 a DNA fragment containing the control region of the *E. coli* tryptophan operon, and coding for the ribosome binding site and first seven amino acids of trpE. [....] The [FPV haemagglutinin protein] protein resulting from initiation at the trpE AUG would be a hybrid [....] It was also clear that the HA gene should not be expressed from the initiator AUG of the trpE fragment in pWT111 or pWT131. Recognition of the ribosome binding site and initiator AUG of the HA was not anticipated.

and;

> In this case [pWT111/FPV502(R)] the HA gene is in the wrong phase to be translated from the trpE AUG; protein synthesis initiating at the trpE AUG is terminated by the now in phase UGA triplet at position 23-25 in the HA gene. Thus we conclude that initiation is from the natural HA AUG at position 22-24 (Fig. 1A).

(Tab Z at p. 171, lt. col. 4th ¶ to rt. col., 2nd ¶; and at p. 173, lt. col., last ¶.) In this paper, the authors found that the HA gene was directly expressed from the AUG of the HA gene.

27. Thus, in view of the papers discussed above in paragraphs 11-26, one of skill in the art as of March 19, 1980 would have at least known that mammalian proteins can be expressed in bacteria while: (1) maintaining enzymatic function, (2) accumulating in significant levels, and (3) be directly expressed from heterologous control elements, i.e., a bacterial promoter and a hybrid ribosomal binding site.

Scope and Content of the Prior Art: General Methods for Expressing Recombinant Proteins in Bacteria as of March 19, 1980

28. With respect to publications disclosing general methods for producing recombinant proteins in bacteria, I have reviewed:

Tab E: Backman and Ptashne, Cell, Vol. 13, pp. 65-71, (Jan. 1978);

Tab AA: Roberts et al., Proc. Natl. Acad. Sci. USA, Vol. 76, No. 2, pp. 760-764 (Feb. 1979);

Tab BB: Ptashne et al., U.S. Patent No. 4,332,892, issued June 1, 1982, filed Jan. 10, 1980, which is a continuation of U.S. Serial No. 3,102, filed Jan. 15, 1979, now abandoned;

Tab CC: Ptashne et al., U.S. Patent No. 4,418,149;

Tab DD: Goeddel et al., U.S. Patent No. 4,342,832;

Tab EE: Itakura and Riggs, U.K. Patent Application 2007676;

Tab FF: Gilbert et al., European Patent Application 79301054.7; and

Tab F: Gilbert and Villa-Komaroff, Sci. Am. 242(4):74-94 (Apr. 1980).

29. In my opinion, Tabs E, AA, BB, CC, and DD provide teachings, prior to March 19, 1980, on how to express a eukaryotic gene in bacteria without having to make a bacterial-eukaryotic protein fusion. References E, AA, BB, and CC teach the 'direct' (*i.e.,* expression of the gene without having to fuse the gene in frame downstream of an N-terminus bacterial coding sequence) expression of a gene in bacteria by placing upstream of the gene a ribosome binding site and a bacterial promoter.

30. I have been informed that a U.S. Patent is considered "prior art" or publicly available for considerations under 35 U.S.C. §§ 102 or 103 as of its priority date. I have been told that because U.S. Patent No. 4,332,892 is a continuation of U.S. Serial No. 3,102, and because U.S. Patent No. 4,342,832 is a divisional of U.S. Patent No. 4,332,892, both patents have a priority date of January 15, 1979.

31. In the January 1978 Backman and Ptashne paper, the authors show that:

> λ repressor synthesis is driven wholly or predominantly by the inserted *lac* promoter(s). In our most potent [gene, not protein] fusion, we created a 'hybrid' ribosome binding site; the *lac* promoter and adjacent sequences were positioned so that the *lac Z* gene SD [Shine-Delgarno] sequence was 8 base pairs upstream from the ATG of the *c*I gene. In principle, this strategy should elicit high levels of expression in E. coli of any gene, whatever its source.

(Tab E at p. 65, Abstract.) Thus, not only does this paper teach that proteins can be directly expressed from a *lac* promoter and a hybrid ribosomal binding site, but also that the hybrid ribosomal binding site can be created by positioning the ATG of the gene to be expressed 8 base pairs downstream of the *lac Z* Shine-Delgarno sequence.

32. In the February 1979 Roberts et al. paper, my colleagues and I further developed the method of gene expression taught in the 1978 Backman and Ptashne paper. The method was further developed by determining what distance the *lac* promoter could be from the translational start site of the gene to be expressed (in the paper, the *cro* gene of phage λ). As we stated,

> In this communication, we describe a method that, in principle, will allow the same *lac* promoter fragment to be placed at virtually any distance in front of a gene. The promoter fragment does not encode a translational start. It does, however, encode a sequence required for binding the message to the ribosome (see *Discussion*). Therefore, our gene-promoter fusions will produce a native protein rather than a fusion protein carrying foreign amino-terminal amino acids.

(Tab AA at p. 760, lt. col., 2nd ¶, emphasis added.) As emphasized, the paper teaches that "native" proteins unaccompanied by bacterial peptides can be expressed. The expression of the native protein depends on positioning its start codon, i.e, its ATG or translational start site, downstream of a portable bacterial promoter, i.e., *lac* promoter. Further, the ATG is present as a gene fusion with the *lacZ* Shine-Delgarno sequence, thereby making a "hybrid ribosome binding site." The paper teaches that even though most of the coding region of the leader sequence of *lacZ* is present, translation begins at the ATG of the cloned gene, and efficient expression may be dependent on how far the ATG is separated from the *lac* Shine-Delgarno sequence:

> The *lac* promoter fragment used in the construction of all the plasmids described here contains, in addition to the binding site for RNA polymerase and the start-point of transcription, the coding region for most of the leader of the *lacZ* message including its Shine-Delgarno sequence. Thus, in pTR182 and pTR190, the *lac* Shine-Delgarno sequence is brought into close proximity with the ATG of *cro*. We know that such hybrid ribosome binding sites can be extremely effective from the results with pKB280 [*citing* the 1978 Backman and Ptashne paper; Tab E]. It is possible that the separation of this sequence and the ATG in pTR190 and pTR182 is not optimum - the *lac* sequence is separated from the ATG of *cro* by 10 bases in pTR182 and by 5 bases in pTR190, whereas the same sequence is separated from the ATG of the *cI* gene by 8 bases in pKB280 and is separated from the ATG of the *lacZ* gene by 7 bases in the *E. coli* chromosome.

(Id. at p. 764, lt. col., 1st ¶.) The paper also teaches "exonuclease III and S1 nuclease digestion used here should allow the placement of the promoter-containing fragment at virtually any distance upstream from most other genes. [....] In particular, the technique should be useful in positioning the *lac* promoter fragment adjacent to a eukaryotic gene with the hope of forming a hybrid ribosome binding site from the *lac* Shine-Delgarno sequence and the ATG of the eukaryotic gene." (Id. at p. 764, lt. col., 2nd ¶.)

33. In U.S. Patent 4,332,892, the disclosure of which was filed on Jan. 15, 1979, the applicants (which originally included myself) describe an invention that is "a process to produce specific proteins coded for by eukaryotic (or prokaryotic) DNA in bacteria. The invention, which uses recombinant DNA techniques, produces proteins in their natural, functional state unencumbered by extraneous peptides." (Tab BB at Abstract.) As in my February 1979 paper (Tab AA), the patent teaches that protein expression is controlled by positioning the protein's ATG downstream of the *lac* Shine-Delgarno site such that this provides a hybrid-ribosome binding site, which is itself downstream of the *lac* promoter, where the promoter controls transcription:

> [N]ucleases, restriction enzymes, and DNA ligase to position a portable promoter consisting of a DNA fragment containing a transcription site but no translation initiation site near the beginning of the gene which codes for the desired protein to form a hybrid ribosomal binding site. The protein produced by the bacterium from this hybrid is the native derivative of the implanted gene. It has been found that the endonuclease digestion product of the *E. coli lac* operon, a fragment of DNA which contains a transcription initiation site but no translational start site, has the required properties to function as a portable promoter in the present invention, being transcribed at high efficiency by bacterial RNA polymerase. The mRNA produced contains a Shine-Delgarno (S-D) Sequence but it does not include the AUG or GUG required for translational initiation. However, in accordance with the present invention, a hybrid ribosomal binding site is formed consisting of the S-D sequence and initiator from the lac operon and the ATG of the gene, and such a fused gene is translated and transcribed efficiently. Using the enyzmes exonuclease III and S1, the promoter may be put at any desired position in front of the translational start site of the gene in order to obtain optimum production of protein. Since the promoter can be inserted at a restriction site ahead of the translational start site of the gene, the gene can first be cut at the restriction site, the desired number of base pairs and any single stranded tails can be removed by treating with nucleases for the appropriate time period, and religating.

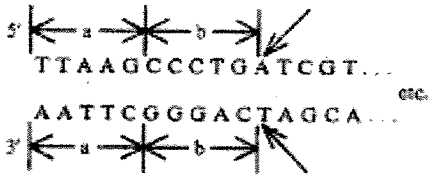
(Tab BB at col. 1, l. 49 to col. 2, l. 11.) This passage provides detailed teachings regarding how to directly express a mature human interferon protein without any leader sequence in bacteria. First, the full-length cDNA can be cut at a restriction site upstream of the mature coding sequence ATG (ATG coding for residue 1 of amino acids 1-166 listed in Fig. 2 of the Taniguchi

Gene paper (Tab LL) or in Fig. 1 of the Taniguchi Preprint (Tab MM)), *i.e.,* "the gene can first be cut at the restriction site." Exonuclease III can then be used to digest away the presequence and any other upstream sequence, S1 to remove any single stranded tails, and ligase to rejoin the DNA, *i.e.,* "the desired number of base pairs and any single stranded tails can be removed by treating with nucleases for the appropriate time period, and religating." In this manner, the ATG of the presequence and all other unwanted bases of the presequence can be removed, such that the (first) ATG coding for the mature interferon protein can form a hybrid ribosomal binding site with the Shine-Delgarno sequence, *i.e.,* "a hybrid ribosomal binding site is formed consisting of the S-D sequence and initiator from the lac operon and the ATG of the gene." Afterwhich, "the promoter may be put at any desired position in front of the translational start site of the gene in order to obtain optimum production of protein."

34. U.S. Patent No. 4,418,149 (Tab CC) is identical to U.S. Patent No. 4,332,892, except for the claims.

35. The Goeddel et al. U.S. Patent No. 4,342,832 (Tab DD), filed July 5, 1979, also reports the expression of proteins in bacteria without making a fusion protein. Similar to the "Ptashne lab methods" (Tabs E, AA, Y, BB, i.e., Backman and Ptashne, Cell, Vol. 13, pp. 65-71, (Jan. 1978); Roberts et al., Proc. Natl. Acad. Sci. USA, Vol. 76, No. 2, pp. 760-764 (Feb. 1979); Roberts et al., Proc. Natl. Acad. Sci. USA, 76:5596-5600 (Nov. 1979), Ptashne et al., U.S. Patent No. 4,332,892) this patent teaches that translation begins at an ATG of an inserted gene, where the ATG start signal can be "the same distance from the ribosome binding site [codons] of the lac promoter as occurs in nature between those [codons] and the start signal for β-galactosidase." (Tab DD at col. 11., ll. 49-53.) The Goeddel patent reports that unwanted sequence between the

ATG and the bacterial control elements (i.e., promoter and S-D sequence) can be removed by digestion:

> Thus, in any cDNA coding for the desired polypeptide and a leader or other bioinactivating sequence the boundary between the latter's codons and those of the mature polypeptide will appear from the amino acid sequence of the mature polypeptide. One may simply digest into the gene coding of the peptide of choice, removing the unwanted leader or other sequence. Thus, for example, given the cDNA such as
>
> 
>
>
> where the endpoint of digestion is indicated by arrow, reaction conditions for exonuclease digestion may be chosen to remove the upper sequences "a" and "b", whereafter S1 nuclease digestion will automatically eliminate the lower sequences "c[a]" and "d[b]".

(Id. at col. 5, l. 60 to col. 6, l. 15.) The above passage explicitly teaches that a leader sequence can be removed by exonuclease/S1 digestion.

36. Itakura and Riggs, U.K. Patent Application 2007676 (Tab EE); Gilbert et al., European Patent Application 79301054.7 (Tab FF); and Gilbert and Villa-Komaroff, Sci. Am. 242(4):74-94 (Apr. 1980)(Tab F), teach methods for expressing recombinant proteins in bacteria by making fusion proteins.

Scope and Content of the Prior Art: General Methods for Recombinant DNA Cloning as of March 19, 1980

37. I have reviewed the following papers that describe general methods for recombinant DNA cloning and screening of expressed recombinant DNA as of March 19, 1980:

Tab GG: Curtiss, Ann. Rev. Microbiol., 30:507-33 (1976);

Tab HH: Wu and Bahl, Prog. Nucleic Acid Res. Mol. Biol. 21:101-41 (1978); and

Tab II. Erlich et al., Cell, Vol. 13, pp. 681-689 (1978).

38. These papers supplement the cloning methods described in the above described prior art.

39. The Curtiss paper (Tab GG) states that it "has recently become possible to enzymatically couple viral, procaryotic, or eucaryotic DNA to bacteriophage or plasmid-cloning vector DNA and to introduce these recombinant DNA molecules into bacteria to clone and ultimately to allow study of the expression of the foreign genetic information." (Tab GG at p. 507 last ¶ to p. 508, 1st ¶.) Specifically, the paper teaches:

> In order to prepare recombinant DNA molecules in vitro, DNA is isolated from an organism of choice, either with or without suitable enrichment for specific gene sequences, and then one of two methods is used to construct recombinant DNA molecules. In one procedure, the DNA is fragmented either enzymatically or by shearing, then treated with an exonuclease that digests a single DNA strand in the 5' to 3' direction; then single-stranded homo-deoxypolymeric sequences are added to the 3' single-stranded ends of the molecule by using a suitable deoxyribonucleotide triphosphate (dXTP) species and terminal deoxynucleotidyl transferase. The cloning vector DNA is similarly cleaved and treated with exonuclease, then complementary homodeoxy-polymeric sequences are added by use of terminal transferase. The two DNA fractions are permitted to anneal and then may be treated with exonuclease III, DNA polymerase I, and polynucleotide-joining enzyme (DNA ligase) to form covalently closed circular recombinant DNA molecules. In another procedure, the DNA is fragmented by using a restriction endonuclease which generates short complementary cohesive ends at both termini of the DNA duplex. The cloning vector DNA is treated with the same enzyme, the two preparations are mixed to permit annealing, and ligase is added to yield covalently closed circular recombinant DNA molecules.

(Tab GG at p. 508, 2nd ¶.) These cloning methods are similar to those described by the Ptashne papers (Tabs E, Y, AA, BB) or the Goeddel '832 patent (Tab DD). As cited above in paragraph 33, the Ptashne '832 patent teaches that exonuclease III and S1 nuclease can be used to remove unwanted base pairs, such as nucleotides in the human fibroblast interferon presequence, "the promoter can be inserted at a restriction site ahead of the translational start site of the gene, the gene can first be cut at the restriction site, the desired number of base pairs and any single stranded tails can be removed by treating with nucleases for the appropriate time period, and

religating." Also, as cited above in paragraph 35, the Goeddel '832 patent reports "in any cDNA coding for the desired polypeptide and a leader or other bioinactivating sequence the boundary between the latter's codons and those of the mature polypeptide will appear from the amino acid sequence of the mature polypeptide. One may simply digest into the gene coding of the peptide of choice, removing the unwanted leader or other sequence."

40. The Wu et al. paper (Tab HH) states that the "chemical synthesis of oligodeoxynucleotides when used in conjunction with enzymic methods permits the preparation of longer segments and sequence variations with minimal effort." (Tab HH at p. 108, 1st ¶.) The paper reports methods as to, for example, chemical synthesis of oligodeoxyribonucleotides, enzymatic synthesis or joining of oligodeoxynucleotides, sequence analysis, synthetic oligonculeotides as tools in molecular cloning of DNA.

41. The Erlich et al. paper (Tab II) teaches an immunoassay for detecting products translated from cloned DNA fragments. Immunoassays will indicate that a particular assayed extract may contain a protein having the correct conformation such that an antibody to that protein will detect it.

One of Ordinary Skill in the Art as of March 19, 1980

42. In my opinion, a person of ordinary skill in the field of recombinant DNA technology or bacterial protein expression as of March 19, 1980 would have a Ph.D. degree or an equivalent degree, or be a highly skilled pre-doctoral fellow, with several years of research experience in molecular biology.

Differences Between the Prior Art and the Goeddel Claims

43. I have reviewed Goeddel U.S. Application Serial No. 07/374,311 (Tab RR) and its involved claims in Interference No. 105,334 (Tab JJ). I have reviewed Goeddel U.S. Patent

No. 5,460,811 (Tab UU) and its involved claims in Interference No. 105,337 (Tab KK). The subject matter of the Goeddel claims in these interferences is disclosed in the prior art: (1) the disclosure of the human fibroblast interferon cDNA sequence and encoded amino acid sequence, (2) a method of expressing such a cDNA sequence by positioning the ATG of the mature human fibroblast coding sequence downstream of the *lac* promoter and Shine-Delgarno site, and (3) a fully non-glycosylated human fibroblast interferon. With respect to (1), the Taniguchi Gene paper or Preprint provides the sequence. With respect to (2), the Ptashne lab prior art [see paragraphs 25, 31-33], and U.S. Patent No. 4,342,832 [paragraph 35] clearly contain detailed teachings of how to carry-out a method for direct expression in bacteria. With respect to (3), proteins produced in E. coli are necessarily non-glycosylated.

44. As for the human fibroblast interferon cDNA sequence, I have been informed that the Taniguchi et al., Gene (May 19, 1980) paper (hereafter referred to as "the Taniguchi Gene paper"; Tab LL) is prior art at least as of June 2, 1980. I have reviewed this paper, and it discloses the interferon sequence. In fact, the paper explicitly discloses where the leader or presequence ends, and where the mature sequence begins, "the 5' terminal region of the interferon mRNA, as deduced from the cDNA, contains at least 6 nucleotides before the first AUG codon in phase. If this AUG functions as an initiator, the primary translation product of human fibroblast interferon mRNA contains a signal peptide consisting of 21 amino acids which would eventually be cleaved off during secretion." (Tab LL at p. 12, rt. col., 2nd ¶.) Thus, as stated in the legend to Figure 2, the "DNA sequence permits prediction of the entire amino acid sequence (for human fibroblast interferon (amino acids 1-166) and its putative signal peptide (amino acids -21 to -1) as shown in the line above the DNA sequences." (Id. at p. 13.) In my opinion, given the Ptashne lab methods as disclosed in Tabs E, Y, AA, BB, a person of ordinary

skill in the art as of May 19, 1980[1] would only require routine experimentation to (1) insert the interferon cDNA into a bacterial plasmid, such as pBR322, (2) linearize this plasmid with a restriction enzyme upstream of the cDNA, (3) digest the cDNA with timed exonuclease III and S1 digestions, or other alternate nucleases, in order to remove the ATG of the leader sequence (i.e., residue -21 in Figure 2 of Tab LL) and other unwanted nucleotides, (4) insert the *lac* promoter and Shine-Delgarno sequence upstream of the now digested cDNA, such that transcription is controlled by the *lac* promoter and translation is initiated at what is now the first ATG downstream of the Shine-Delgarno sequence, i.e., residue 1 of Figure 2 of Tab LL).

45. I also understand that the preprint entitled "Human leukocyte and fibroblast interferons are structurally related," authored by Tadatsugu Taniguchi, Charles Weissmann and others, (Tab MM (hereafter referred to as the "Taniguchi Preprint")), is prior art at least as of April 8, 1980. I have reviewed this preprint, and the preprint also discloses the cDNA of human

[1] Or as of the date when the human fibroblast interferon cDNA would be available, such as March 19, 1980 with respect to the sequence provided by Japanese Application No. 55-33931 (certified translation is Tab NN). The Japanese Application No. 55-33931cites Knight, et al. [Science vol. 207, p. 525-526, (1980); Tab OO]. The Knight paper discloses the sequence of the N-terminal 13 amino acids of the secreted or mature form of human fibroblast interferon. In view of Knight's disclosure of the sequence of the N-terminal 13 amino acids of human fibroblast interferon, a person of ordinary skill in the field of recombinant DNA technology or bacterial protein expression as of March 19, 1980 would have immediately known the coding sequence for the mature human fibroblast interferon amino acid sequence given within the full-length cDNA sequence. Page 15 of the Japanese application lists the nucleotide sequence of the human fibroblast interferon cDNA and encoded amino acids. The amino acid sequence contains the leader or presequence of interferon as well as the mature protein sequence, but does not explicitly demarcate where the presequence ends and where the mature protein sequence begins. But in view of the Knight disclosures, one of ordinary skill would have immediately understood that the presequence consists of the first 21 amino acids because the Knight disclosures teach that the mature sequence begins with the amino acid sequence Met-Ser-Tyr-Asn-Leu-Leu-Gly-Phe-Leu-Gln-Arg-Ser-Ser, which corresponds to amino acids 22-34 listed at page 15 of the Japanese Patent Application (see Tab NN at page 15). In fact, the Japanese Patent Application explicitly states at page 16: "It is important that in the sequence there exist without any errors the base sequence [three base pairs] corresponding to the amino acid sequence from the amino-terminal to the 13th amino acid of the human fibroblast interferon reported by Knight, et al. [Tab OO at p. 525-526, (1980)]."

fibroblast interferon, and where the presequence or leader sequence ends (S21) and where the mature sequence begins (residue 1). (See Figure 1, Figure 1 legend, and p. 3, ll. 4-16 of Tab MM.) Thus, in my opinion, given the prior art, such as the Ptashne lab methods as disclosed in Tabs E, Y, AA, BB, a person of ordinary skill in the art as of April 8, 1980 would only need routine experimentation to (1) insert the interferon cDNA into a bacterial plasmid, such as pBR322, (2) linearize this plasmid with a restriction enzyme upstream of the cDNA, (3) digest the cDNA with timed exonuclease III and S1 or other nuclease digestions in order to remove the ATG of the leader sequence (i.e., residue S1 in Figure 1 of Tab MM) and other unwanted base pairs, (4) insert upstream of the *lac* promoter and Shine-Delgarno sequence of the now digested cDNA, such that transcription is controlled by the *lac* promoter and translation is initiated at what is now the first ATG downstream of the Shine-Delgarno sequence, i.e., residue 1 of Figure 1 of Tab MM).

46. I have been informed that routine experimentation does not preclude complex experimentation or a large quantity of experimentation. Rather, I have been informed that routine experimentation can entail repetitive experimentation if the art typically engages in such experimentation. Thus, to make a human fibroblast interferon cDNA without any leader sequence, the cDNA can be digested with exonuclease III and S1 or other nucleases in order to generate a number of clones, one of which would have all of its presequence digested up to the mature ATG sequence. In fact, Goeddel's U.S. Patent No. 4,342,832 explicitly teaches that exonuclease III and S1 can be used to remove leader sequences. After digestion, the clones are religated, transformed into bacteria, and plasmid DNA can then be purified and analyzed, for example, by acrylamide gel analysis. Acyrlamide gel analysis will allow the practitioner to determine which clones might have the entire presequence digested away. These clones can then

be sequenced to confirm which ones only have the coding sequence for the mature interferon, i.e., without coding sequence for the presequence. Alternatively, protein extracts from bacterial clones can be tested by immunoassays to identify potential clones that express the protein. Plasmids from these potential clones can be purified and sequenced to confirm which plasmids contain only the coding sequence for the mature interferon. Although this process can be labor intensive, the necessary methods (exonuclease and S1 digestion, restriction digests, ligation, and sequencing (see ¶¶ 37-41 above)) were well-established in the art at least as of March 19, 1980. Further, it was known in the art at least by Feb. 2, 1980 (Knight, et al., Science, Vol. 207, p. 525-526, (1980)), that the secreted, functional form of the human fibroblast interferon protein has an N-terminal sequence of sequence Met-Ser-Tyr-Asn-Leu-Leu-Gly-Phe-Leu-Gln-Arg-Ser-Ser. Thus, in light of this N-terminal sequence information, given the human fibroblast cDNA sequence provided by the Taniguchi Gene paper or Taniguchi Preprint, one of ordinary skill in the art would have known the boundary between the presequence and the coding sequence for the mature interferon (*i.e.,* the secreted functional form) and would desire to make a DNA coding for a mature interferon without presequence because this has been shown to be the secreted, native, functional form of human fibroblast interferon. Further, in view of the Ptashne lab methods (Tabs E, Y, AA, BB) and other known methods of mammalian protein expression in bacteria, one of ordinary skill in the art could make, without undue experimentation, a bacterial expression plasmid that directs the transcription and translation of the mature human fibroblast interferon protein through the formation of a hybrid ribosomal binding site with the ATG of the mature interferon sequence and the placement of the *lac* promoter upstream of the hybrid ribosomal binding site.

Motivation to Combine

47. I have been informed that for a combination of prior art references to make an invention obvious, there must be some suggestion to those of ordinary skill in the art to actually combine the particular combination of prior art references. I have been informed that the suggestion to combine need not be found explicitly in the references sought to be combined. Rather, the suggestion may be implicitly found from the prior art as whole, including common knowledge, or the nature of the problem to be solved. However, I have been informed that it is improper to pick and choose among isolated disclosures in the prior art to deprecate the claimed invention.

48. In my opinion, one of ordinary skill in the art as of March 19, 1980 (with respect to Japanese Application No. 55-33931), May 19, 1980 (with respect to the Taniguchi Gene paper) or April 8, 1980 (with respect to the Taniguchi Preprint), would have been motivated to combine the Ptashne 4,332,892 patent (Tab BB; hereafter referred to as "the Ptashne '892 patent") with either the Taniguchi Preprint or the Taniguchi Gene paper, because the motivation is found explicitly in the Ptashne patent or from the nature of the problem of mass-producing interferon. For example, the Ptashne patent explicitly states that its invention is "a process to produce specific proteins coded for by eukaryotic (or prokaryotic) DNA in bacteria." (Tab BB at Abstract.) The human fibroblast interferon cDNA is eukaryotic DNA. Further, the Ptashne patent explicitly states that its invention can be used to produce a native or mature human fibroblast interferon (*i.e.*, without presequence and without being fused to a bacterial protein):

> Recombinant DNA techniques in vitro have been used to insert a variety of eukaryotic genes into plasmids carried by *Escherichia coli* in an effort to induce these bacteria to produce eukaryotic proteins. Most of these genes have not directed the synthesis of the native proteins [. . . .] The present invention provides a method of producing native, unfused prokaryotic or eukaryotic protein in bacteria ...

(Tab BB at col. 1, ll. 12-37.) One of ordinary skill would have also been motivated to combine these references because of the nature of the problem of mass-producing interferon and because of the explicit suggestion to produce proteins in bacteria. As discussed above in paragraphs 6-9, the prior art explicitly discussed this linkage (*i.e.*, the problem of mass production and the solution of bacterial production) in the July 1979 Anonymous Research Disclosure entitled "The production of interferon by 'genetic engineering,'" (Tab D; hereafter referred to as the "Anonymous Research Disclosure"):

> Human interferon is usually produced from either leukocytes or from fibroblast cells. Up to now, clinical studies have employed human leukocyte interferon. One cell is able to make only minute quantities of interferon so large quantities of human cells are needed for production. The problem of shortage of human interferon for clinical studies is still the major drawback. It is for this reason that some people have turned their attention to a line of transformed human lymphblastoid cells. These cells can be grown in very large numbers and hence larger quantities of interferon can be produced. However, probably the best way to produce large quantities of interferon will be to introduce the gene(s) for interferon production into bacteria. This technique has already been demonstrated with other mammalian genes eg somatostatin, insulin, ovalbumin, dihydrofolate reductase and hepatitis antigen.

(See Tab D at first page, 2nd ¶.) Thus, because the Anonymous Research Disclosure explicitly states the nature of the problem and the solution of interferon production in bacteria, the motivation to combine the Anonymous Research Disclosure with the Ptashne '892 patent, and the Taniguchi Gene paper or the Taniguchi Preprint is thereby explicitly provided.

49. Likewise, in my opinion, one of ordinary skill in the art would have been motivated to further combine the Ptashne '892 patent and the Taniguchi Gene paper or Preprint with the Goeddel Nature (Oct. 1979) paper (Tab U; hereafter referred to as the "Goeddel Nature paper"). The motivation to further combine with the Goeddel paper is explicit within this paper: "The hybrid DNA cloning techniques described as a route to the cloning and expression of HGH coding sequences in *E. coli* are generally applicable to other polypeptides which are synthesised

initially as inactive precursors and later processed, or for which full length cDNA transcripts are unavailable." (Tab U, emphasis added, at p. 548, rt. col., 4th ¶.)

50. Further, one of ordinary skill in the art would have been motivated to further combine with the Fujisawa paper (Tab PP; hereafter referred to as the "Fujisawa paper") because this paper discusses whether interferons are functional without glycoyslation. The issue of functionality is relevant with respect to the above-mentioned problem of interferon mass production, because interferon mass production was desired for therapeutic application.

Reasonable Expectation of Success

51. I have been informed that for the prior art to make an invention obvious, one of ordinary skill in the art must have a reasonable expectation of success for making the invention given the prior art. I have been informed that a reasonable expectation of success is to be assessed from the perspective of one of ordinary skill in the art at the time the invention was made. I have also been informed that a reasonable expectation of success is present in the prior art where the prior art contains a detailed enabling methodology for practicing the claimed invention, a suggestion to modify the prior art to practice the claimed invention, and evidence suggesting that it would be successful. I have been informed that a reasonable expectation of success does not require absolute predictability, but rather a reasonable likelihood of success.

52. In my opinion, one of ordinary skill in the art as of March 19, 1980 (with respect to Japanese Application No. 55-33931), May 19, 1980 (with respect to the Taniguchi Gene paper) or April 8, 1980 (with respect to the Taniguchi Preprint), would have had a reasonable expectation of successfully making: (1) recombinant DNA molecules consisting of a coding sequence for the mature human fibroblast interferon (*i.e.*, the nucleotide sequence coding for amino acids 1-166 as displayed in Figure 2 of the Taniguchi Gene paper (Tab LL), or as

displayed in Figure 1 of the Taniguchi Preprint (Tab MM), or as displayed on page 15 of the translation of Japanese Application No. 55-33931 (Tab OO); (2) bacterial expression constructs capable of expressing the mature human fibroblast interferon; and (3) the mature human fibroblast interferon protein having amino acids 1-166 unaccompanied by presequence or other sequence.

53. First, one of ordinary skill in the art as of as of March 19, 1980, April 8, 1980, or May 19, 1980, would have had a reasonable expectation of successfully making the molecules described in paragraph 52 above because the Ptashne '892 patent (and other Ptashne lab papers [see paragraphs 23, 31-33]) contains a detailed enabling methodology to make these molecules. As stated above in paragraphs 43-44, the Ptashne patent in view of the human fibroblast interferon sequences disclosed in the Taniguchi Gene or Preprint papers teaches how to: (1) insert the interferon cDNA into a bacterial plasmid, such as pBR322, (2) linearize this plasmid with a restriction enzyme upstream of the cDNA, (3) digest the cDNA with timed exonuclease III and S1 digestions in order to remove the ATG of the leader sequence (i.e., residue -21 in Figure 2 of Tab MM or residue S1 in Figure 1 of Tab LL) and other unnecessary nucleotides, (4) insert upstream of the now digested cDNA the *lac* promoter and Shine-Delgarno sequence, such that transcription is controlled by the *lac* promoter and translation is initiated at what is now the first ATG downstream of the Shine-Delgarno sequence, (i.e., residue 1 of Figure 2 of Tab LL or Figure 1 of Tab MM). For example, see paragraph 33 above, which provides a passage from the Ptashne patent that provides explicit instructions. Further, see paragraph 35 above, which provides a passage from the Goeddel Nature paper that provides similar explicit instructions. The cloning techniques described in the Ptashne and Goeddel disclosures were well known in the

prior art, as similar techniques were also described in the Curtiss and Wu papers (see paragraphs 39-40 above).

54. Second, one of ordinary skill in the art as of March 19, 1980, April 8, 1980, or May 19, 1980, would have had a reasonable expectation of successfully making the molecules described in paragraph 52 above because the Ptashne '892 patent, Goeddel Nature paper, and the Anonymous Research Disclosure all provide explicit suggestions to modify the prior art and practice the claimed inventions of Goeddel (see paragraphs 47-49 above).

55. Third, one of ordinary skill in the art as of as of March 19, 1980, April 8, 1980, or May 19, 1980, would have had a reasonable expectation of successfully making the molecules described in paragraph 52 above because there is ample evidence in the prior art that making such molecules would be successful. For example: (1) the August 1977 Vapnek article shows that control of transcription of a eukaryotic gene in bacteria can take place by a bacterial promoter (see paragraph 12 above); (2) the December 1977 Itakura paper shows the successful transcription and translation of a chemically synthesized gene in bacteria (see paragraph 13 above); (3) the August 1978 Villa-Komaroff paper shows the successful reproduction of a mammalian protein's structure by bacterial machinery (see paragraph 14 above); (4) the October 1978 Chang paper shows the successful expression of a functional mammalian protein in bacteria (see paragraph 15 above); (5) the October 1978 Mercereau-Puijalon, the December 1978 Seeburg, and the August 1979 Martial publications show the successful expression of long polypeptides from higher eukaryotes in bacteria (see paragraphs 16, 18, and 22 above); (6) the December 1978 Fraser paper shows that eukaryotic proteins can be stable in the bacterial intracellular environment (see paragraph 17 above); (7) the Goeddel October 1979 paper show that eukaryotic proteins can be directly expressed in bacteria without presequence (see paragraph

19 above); (8) the Emtage 1980 Nature paper shows that a eukaryotic protein can be directly expressed; and (9) the Roberts November 1979 paper shows the successful practice of the method described in the Ptashne '892 patent, where a eukaryotic protein is expressed in bacteria without extraneous peptides, directly from the eukaryotic protein's ATG, and directly from bacterial control elements (see paragraph 25 above). Further, the Ptashne '892 patent provides an example of its invention where bacterial expression constructs containing the rabbit β-globin gene were designed. This example was built upon (at the 3' end of a 5' portion of the β-globin gene, lacZ genes were fused in order to provide an efficient method for screening, where positive clones were isolated such that the full-length β-globin gene was remade and then expressed in bacteria in a non-fusion form) and later successfully conducted and published. (See Tab QQ, "Improved Methods for Maximizing Expression of a Cloned Gene: a Bacterium that Synthesizes Rabbit β-Globin," published June 1980, received March 10, 1980). Th Ptashne '892 Example explicitly stated that the "globin gene in the above construction can be any gene coding for prokaryotic or eukaryotic proteins." (Tab BB at col. 2, ll. 48-49.)

Disclosure of the Goeddel Claims in the Prior Art:

A. Goeddel U.S. Application Serial No. 07/374,311 ('311 application) claims 25, 26, 34-40, 42-47 are anticipated by the Taniguchi Gene paper or the Taniguchi Preprint:

56. I have been informed that for a claim to be anticipated by the prior art, a single prior art disclosure must contain all of the elements of the claim, either explicitly or inherently. I have been informed that inherent disclosure means that the element is necessarily disclosed in the paper, but that one of ordinary skill in the art at the time of the prior art disclosure need not know that the element is necessarily disclosed.

All of the elements of claim 25 are disclosed in either the Taniguchi Preprint or Gene paper:

a. Claim 25 element "A DNA encoding a mature human fibroblast interferon having a total of 166 amino acids," is explicitly disclosed:

i. At p. 13, Fig. 2 and Fig. 2 Legend of the Taniguchi Gene paper: "Nucleotide sequence of the human fibroblast interferon cDNA and the predicted amino acid sequence of the pre-interferon molecule. ... The DNA sequence permits prediction of the entire amino acid sequence for human fibroblast interferon (amino acids 1-166) and its putative signal peptide (amino acids -21 to -1) as shown in the line above the DNA sequences" (underlining added); and

ii. At Legend for Fig. 1 of the Preprint: "Comparison of the nucleotide sequences of human leukocyte interferon I (Le-IF I) and human fibroblast interferon cDNA and of the derived amino acid sequences. The sequences are from Mantei et al. (19) and Taniguchi et al. (21). They were aligned to give maximal homology. Identical amino acids are framed, identical nucleotides are marked by a dot. S1 to S23 indicate the amino acids of the putative signal sequence; 1 to 166 the amino acids of the interferon polypeptides." (Underlining added.)

b. Claim 25 element "and unaccompanied by a human fibroblast interferon presequence" is inherently disclosed:

i. At p. 13, Fig. 2 and Fig. 2 Legend of the Taniguchi Gene paper: "The DNA sequence permits prediction of the entire amino acid sequence for human fibroblast interferon (amino acids 1-166) and its putative signal peptide (amino acids -21 to -1) as shown in the line above the DNA sequences." Because the reference teaches exactly where the signal peptide begins and ends, and because cloning techniques were routine at the time (see paragraphs 33, 35, 39-40), a DNA coding for the mature human fibroblast interferon "unaccompanied" by presequence is inherent to the reference.

ii. At p. 3, ll. 4-16 of the Preprint: "In Fig. 1, the nucleotide sequences of Le-IF I and F-IF cDNA were aligned so that the AUGs closest to their 5' termini coincided. From the amino terminal sequence published for F-IF (16) and lymphoblastoid Le-IF (22) one can determine that in the case of F-IF the 21st codon following the initiation triplet and in the case of Le-IF the 23rd codon represents the first amino acid of the interferon polypeptide." Again, because the reference teaches exactly where the signal peptide begins and ends, and because cloning techniques were routine at the time, a DNA coding for the mature human fibroblast interferon "unaccompanied" by presequence is inherent to the reference.

iii. In my opinion, given the Taniguchi Gene or Preprint disclosure, one of ordinary skill in the art would know how to make a cDNA coding for the mature interferon without presequence and would desire to make such a cDNA in view of the established fact at the time that the native functional interferon is secreted by human cells without presequence.

57. I understand that claim 26 further limits claim 25 by the limitation that the DNA of claim 25 has at least the nucleotide sequence shown in claim 26 of Tab J. This nucleotide sequence, which is the coding sequence for the mature human fibroblast interferon, is disclosed at Fig. 2 of the Taniguchi Gene paper or Fig. 1 of the Taniguchi Preprint. The Taniguchi Gene paper nucleotide sequence at nucleotides 70 to 567 is identical to the nucleotide sequence listed in Goeddel claim 26 (and claims 36 and 42), except for the nucleotides that encode amino acid residue 111 (phenylalanine), where the Taniguchi Gene paper recites "TTC" and the Goeddel claims recite "TTT". Thus, the one nucleotide difference is inconsequential because both triplets code for phenylalanine. The Taniguchi Preprint discloses a cDNA sequence that is identical to the cDNA sequence disclosed by the Taniguchi Gene paper.

58. Claim 34 recites "A DNA which consists essentially of a DNA which codes for human fibroblast interferon polypeptide." I have been informed that "consists essentially of" should be interpreted broadly, and that a DNA which consists essentially of a DNA which codes for human fibroblast interferon polypeptide can include sequence additional to the coding sequence for a mature human fibroblast interferon polypeptide. But even if the claim is interpreted to be limited to a DNA only having the coding sequence for a mature human fibroblast interferon, the Taniguchi Gene paper (at Fig. 2) and the Taniguchi Preprint (at Fig. 1) disclose DNA molecules that code for human fibroblast interferon, either with or without presequence. As discussed above in paragraph 56, both papers are explicit as to where the presequence ends and where the mature sequence begins. Exercising prior art cloning techniques such as those described in paragraphs 33, 35, 39-40, one of ordinary skill in the art could routinely make DNA molecules having a sequence that is limited to the coding sequence for the mature human fibroblast interferon.

59. Claim 35 recites: "A DNA consisting essentially of a DNA which codes for mature human fibroblast interferon polypeptide having the amino acid sequence: [as displayed in claim 36 of Tab JJ; *i.e.*, the amino acid sequence of the mature human fibroblast interferon]. Both the Taniguchi Gene paper (at Fig. 2) and the Taniguchi Preprint (at Fig. 1) disclose DNA molecules that code for human fibroblast interferon, either with or without presequence. As discussed above in paragraph 56, both papers are explicit as to where the presequence ends and where the mature sequence begins. Exercising prior art cloning techniques such as those described in paragraphs 33, 35, 39-40, one of ordinary skill in the art could routinely make DNA molecules having a sequence that is limited to the coding sequence for the mature human fibroblast interferon.

60. Claim 36 recites: "A DNA consisting essentially of a DNA according to claim 35 which has the base pair sequence: [as displayed in claim 36 of Tab JJ; *i.e.*, the nucleotide sequence coding for the amino acid sequence of the mature human fibroblast interferon]. Both the Taniguchi Gene paper (at Fig. 2) and the Taniguchi Preprint (at Fig. 1) disclose DNA molecules that code for human fibroblast interferon, either with or without presequence. As discussed above in paragraphs 56-57, both papers provide the claimed nucleotide sequence and are explicit as to where the presequence ends and where the mature sequence begins. Exercising prior art cloning techniques such as those described in paragraphs 33, 35, 39-40, one of ordinary skill in the art could routinely make DNA molecules having a sequence that is limited to the coding sequence for the mature human fibroblast interferon.

61. I understand that claim 37 limits claim 36 by specifying the DNA of claim 36 is "cloned." Figure 2 of the Taniguchi Gene paper provides the sequence from the "cDNA," which means complementary DNA that is synthesized from mRNA by reverse transcriptase. This necessarily means that a cDNA is cloned, which was cloned into "[h]ybrid plasmid TpIF319-13, consists of a cDNA of about 800 base pairs inserted at the EcoRI site of pBR322." (Tab LL at p. 12, lt. col., last ¶.) The Taniguchi Preprint also provides the sequence from the cDNA, and explicitly mentions that "[w]e have recently cloned and sequenced one species each of Le-IF I) (18, 19) and F-IF cDNA (20, 21)." (Tab MM at p. 2, last ¶.) Again, exercising prior art cloning techniques such as those described in paragraphs 33, 35, 39-40, one of ordinary skill in the art could routinely make DNA molecules having a sequence that is limited to the coding sequence for the mature human fibroblast interferon by, for example, linearizing the plasmid upstream of the cDNA and digesting back the cDNA with exonuclease or other nucleases.

62. Claim 38 recites: "38. A cloned DNA consisting essentially of a DNA which codes for a polypeptide having the amino acid sequence in claim 35." As discussed in paragraph 61 above, both the Taniguchi Gene and Preprint disclose cloned DNA coding for a polypeptide having the mature human fibroblast interferon amino acid sequence.

63. Claim 39 recites: "A recombinant plasmid wherein a DNA which codes for the amino acid sequence: [mature human fibroblast interferon amino acid sequence as listed in at claim 39 in Tab JJ] is inserted in a vector DNA." I understand that claim 39 differs from claim 38 by the limitation "recombinant plasmid." The Taniguchi Gene paper discloses that the cDNA of human fibroblast interferon was cloned into TpIF319-13, which is a recombinant plasmid. The Taniguchi Preprint discloses that the fibroblast interferon cDNA was cloned and referred to reference number 20 (Tab MM at p. 2, last ¶), which is Taniguchi et al., Proc. Jap. Acad. Ser. B, 55, 464-469 (1979)(Tab G), which discloses TpIF319-13. (See Tab G at p. 468, 1st full ¶: "These results thus indicate that the recombinant plasmid DNA from clone no. 319, named TIF319, contains the sequence for the interferon mRNA.")

64. Claim 40 recites: "A microorganism containing the recombinant plasmid defined in claim 39." I understand that claim 40 differs from claim 39 by the limitation "a microorganism" containing the recombinant plasmid of claim 39. The Taniguchi Gene paper states, "a bacterial clone containing the hybrid plasmid TpIF319-13 has been described in previous papers," which previous papers include "Taniguchi et al. 1979," i.e., Taniguchi et al., Proc. Jap. Acad. Ser. B, 55, 464-469 (1979)(Tab G). (See Tab LL at p. 12, lt. col., 2nd ¶.) This paper is the same reference number 20 referred to by the Taniguchi Preprint in its disclosure that the fibroblast interferon cDNA was cloned (Tab MM at p. 2, last ¶).

65. Claim 42 recites: "The recombinant plasmid according to claim 39 wherein said inserted DNA comprises the following base pair sequence: [see nucleotide sequence coding for the mature human fibroblast interferon in Tab JJ]." I understand claim 42 differs from claim 39 by specifying a specific nucleotide sequence coding for the mature human fibroblast interferon amino acid sequence rather than any nucleotide sequence coding for said amino acid sequence. The specific nucleotide sequence listed in claim 42 in Tab JJ is disclosed in Figure 2 of the Taniguchi Gene paper and in Figure 1 of the Taniguchi Preprint, except for the nucleotides that encode amino acid residue 111 (phenylalanine), where the Taniguchi Gene paper recites "TTC" and the Goeddel claims recite "TTT". But this is an inconsequential difference, because both triplets code for phenylalanine. Again, both Taniguchi disclosures are explicit as to where the mature human fibroblast interferon nucleotide and encoded amino acid sequence begins and ends.

66. Claim 43 recites: "The recombinant plasmid according to claim 39 wherein said vector DNA is an Escherichia coli plasmid." I understand that claim 43 differs from claim 39 by specifying that the vector is an E. coli plasmid. Both the Taniguchi Gene paper and Preprint disclose the recombinant plasmid TpIF319-13, which is based on the pBR322 plasmid ("Hybrid plasmid TpIF319-13, consists of a cDNA of about 800 base pairs inserted at the EcoRI site of pBR322," see Tab LL at p. 12, lt. col., last ¶), which is an E. coli plasmid. The Taniguchi Preprint discloses TpIF319-13 because it states that the fibroblast interferon cDNA was cloned and referred to reference number 20 (see Tab MM at p. 2, last ¶), which is Taniguchi et al., Proc. Jap. Acad. Ser. B, 55, 464-469 (1979)(Tab G), which discloses TpIF319-13. (See Tab G at p. 468, 1st full ¶: "These results thus indicate that the recombinant plasmid DNA from clone no. 319, named TIF319, contains the sequence for the interferon mRNA.")

67. Claim 44 recites: "The recombinant plasmid according to claim 43 wherein said Escherichia coli plasmid is pBR322." I understand that claim 44 differs from claim 43 by specifying that the plasmid is "pBR322." As discussed above in claim 43, the TpIF319-13 plasmid is made from the pBR322 plasmid.

68. Claim 45 recites: "A process for preparing a recombinant plasmid which comprises inserting a synthesized double stranded DNA which codes for the amino acid sequence: [mature fibroblast human interferon as shown in claim 45 of Tab JJ] in a vector DNA."

a. The step of "inserting a synthesized double stranded DNA" is disclosed by the Taniguchi Gene reference by its statement that "DNA synthesized by in vitro reverse transcription of the interferon mRNA has been cloned and amplified as recombinant DNA, TpIF319-13 (Taniguchi et al. 1979)." (Tab G at Abstract, emphasis added.) Reverse transcription of interferon mRNA will result in a "synthesized double stranded DNA" which is inserted into a vector DNA because cloning it into TpIF319-13 means it is inserted into a vector DNA. The Taniguchi Preprint also discloses the step "inserting a synthesized double stranded DNA" into "a vector DNA" because the Preprint mentions that "[w]e have recently cloned and sequenced one species each of Le-IF (18, 19) and F-IF cDNA (20, 21)." (Tab MM at p. 2, last ¶.) Cloning the F-IF cDNA in reference 20 involved synthesizing cDNA from interferon mRNA and inserting the synthesized cDNA into pBR322 to generate TpIF319-13.

b. The synthesized double stranded DNA that codes for the mature human fibroblast interferon amino acid sequence is disclosed in the prior art because Fig. 2 of the Taniguchi Gene paper and Fig. 1 of the Preprint present the interferon cDNA sequence and encoded mature amino acid sequence.

69. Claim 46 recites: "The process according to claim 45 wherein said vector DNA is an Escherichia coli plasmid." I understand that claim 46 differs from claim 45 by specifying that the vector DNA is an E. coli plasmid. Both the Taniguchi Gene paper and the Preprint disclose that the vector DNA is an E. coli plasmid because both publications disclose that the interferon cDNA was cloned in TpIF319-13, a pBR322 based E. coli plasmid.

70. Claim 47 recites: "A process according to claim 46 wherein said Escherichia coli plasmid is pBR322." I understand that claim 47 differs from claim 46 by specifying that the E. coli plasmid is pBR322. As stated in paragraph 69 above, TpIF319-13 is a pBR322 based plasmid.

B. Goeddel U.S. Application Serial No. 07/374,311 ('311 application) claims 25-52 are made obvious by the Taniguchi Preprint or the Taniguchi Gene paper in view of the Ptashne '892 patent and the Goeddel Nature (1979) paper:

71. I have been informed that a claim can be made obvious by a combination of prior art disclosures, where the combination discloses either explicitly, implicitly, or inherently all of the elements of the claim.

All of the elements of claim 25 are disclosed in either the Taniguchi Preprint or Gene paper, when combined with the Ptashne '892 patent:

a. Claim 25 element "A DNA encoding a mature human fibroblast interferon having a total of 166 amino acids," is disclosed:

i. At p. 13, Fig. 2 and Fig. 2 Legend of the Taniguchi Gene paper: "Nucleotide sequence of the human fibroblast interferon cDNA and the predicted amino acid sequence of the pre-interferon molecule. ... The DNA sequence permits prediction of the entire amino acid sequence for human fibroblast interferon (amino acids 1-166) and its putative signal peptide (amino acids -21 to -1) as shown in the line above the DNA sequences" (underlining added); and

ii. At Legend for Fig. 1 of the Preprint: "Comparison of the nucleotide sequences of human leukocyte interferon I (Le-IF I) and human fibroblast interferon cDNA and of the derived amino acid sequences. The sequences are from Mantei et al. (19) and Taniguchi et al. (21). They were aligned to give maximal homology. Identical amino acids are framed, identical nucleotides are marked by a dot. S1 to S23 indicate the amino acids of the putative signal sequence; 1 to 166 the amino acids of the interferon polypeptides." (Underlining added.)

b. Claim 25 element "and unaccompanied by a human fibroblast interferon presequence" is disclosed:

i. At p. 13, Fig. 2 and Fig. 2 Legend of the Taniguchi Gene paper: "The DNA sequence permits prediction of the entire amino acid sequence for human fibroblast interferon (amino acids 1-166) and its putative signal peptide (amino acids -21 to -1) as shown in the line above the DNA sequences." The reference teaches exactly where the signal peptide begins and ends.

ii. At p. 3, ll. 4-16 of the Preprint: "In Fig. 1, the nucleotide sequences of Le-IF I and F-IF cDNA were aligned so that the AUGs closest to their 5' termini coincided. From the amino terminal sequence published for F-IF (16) and lymphoblastoid Le-IF (22) one can determine that in the case of F-IF the 21st codon following the initiation triplet and in the case of Le-IF the 23rd codon represents the first amino acid of the interferon polypeptide." Again, the reference teaches exactly where the signal peptide begins and ends.

iii. The Ptashne '892 patent discloses the use of routine cloning techniques to make a DNA coding for the mature human fibroblast interferon "unaccompanied" by presequence:

> [I]n accordance with the present invention, a hybrid ribosomal binding site is formed consisting of the S-D sequence and initiator from the lac operon and the

> ATG of the gene, and such a fused gene is translated and transcribed efficiently. Using the enzymes exonuclease III and S1, the promoter may be put at any desired position in front of the translational start site of the gene in order to obtain optimum production of protein. Since the promoter can be inserted at a restriction site ahead of the translational start site of the gene, the gene can first be cut at the restriction site, the desired number of base pairs and any single stranded tails can be removed by treating with nucleases for the appropriate time period, and religating.

(Tab BB at col. 1, l. 65 to col. 2, l. 11.) To obtain production of the mature interferon, at a minimum, the ATG of the presequence must be removed such that translation begins at the ATG of the mature protein. As taught in the passage above, the gene can first be cut at a restriction site ahead of the ATG of the mature protein and the desired number of base pairs removed. As discussed above in paragraph 46, the entire presequence DNA can be digested away and identification of the desired clone requires only routine experimentation. But if the claim language is interpreted to mean that the DNA can have additional nucleotide sequence as long as the nucleotide sequence only codes for a mature human fibroblast interferon without presequence amino acids, then only the ATG nucleotides of the presequence needs to be digested. Without the ATG nucleotides of the presequence, translation would begin at the next ATG, i.e., the ATG of the mature sequence.

72. I understand that claim 26 further limits claim 25 by the limitation that the DNA of claim 25 has at least the nucleotide sequence shown in claim 26 in Tab JJ. I understand that the DNA of claim 26 can have additional nucleotides over those listed in claim 26 because of the term "comprising." Thus, in view of the Ptashne '892 patent teachings, such as the passage listed in paragraph 71 above, one of ordinary skill in the art would take the Taniguchi Gene or Preprint cDNA and remove the leader sequence ATG in order to have a DNA that encodes a mature human fibroblast interferon without presequence and comprises the nucleotide coding sequence for the mature human fibroblast interferon.

73. Claim 27 recites: "A replicable microbial expression vehicle capable, in a transformant microorganism, of directing expression of a DNA of claim 25 or 26." As discussed above in paragraphs 71-72, the DNA of claim 25 or claim 26 is disclosed by the Taniguchi Gene paper or Preprint, either alone, or in view of the Ptashne '892 patent. The element "a replicable microbial expression vehicle capable in a transformant microorganism of directing expression" of the DNA of claim 25 or claim 26 is disclosed in the Ptashne '892 patent:

> The present invention provides a method of producing native, unfused prokaryotic or eukaryotic protein in bacteria which comprises inserting into a bacterial plasmid a gene for a prokaryotic or eukaryotic protein and a portable promoter consisting of a DNA fragment containing a transcription initiation site recognized by RNA polymerase and containing no protein translational start site, said promoter being inserted ahead of a protein translational start site of said gene to form a fused gene having a hybrid ribosome binding site, inserting said plasmid into said bacteria to transform said bacteria with said plasmid containing said fused gene, and culturing the transformed bacteria to produce said prokaryotic or eukaryotic protein."

(Tab BB at col. 1, ll. 35-48.) This passage teaches an expression plasmid or "vehicle" that directs expression of a native, unfused eukaryotic gene through its a hybrid ribosomal binding site and portable promoter control elements. The passage also teaches that the plasmid is transformed into bacteria, and thus, the passage discloses a "transformant microorganism." The Ptashne '892 patent also explicitly discloses a "replicable" expression plasmid, because it reports the use of pBR322 (see Tab BB at Example at col. 2, l. 18). Thus, all elements of claim 27 are disclosed.

74. Claim 28 recites: "A microorganism transformed with a microbial expression vehicle capable, in said microorganism, of directing expression of a DNA of claim 25 or 26." As discussed above in paragraph 73, the cited passage from the Ptashne '892 patent discloses a microbial expression capable of directing expression of the interferon DNA that is transformed into bacteria.

75. Claim 29 differs from claim 28 by limiting the microorganism to E. coli. The Ptashne '892 patent specifically recites "*Escherichia coli*" at col. 1, l. 14.

76. Claim 30 differs from claim 29 by specifying that the E. coli is "K-12 strain 294." The Goeddel Nature paper states: "Many transformants of E. coli 294 (ref. 16) were obtained with plasmids containing EcoRI and HindIII inserts of approximately the desired size." (Tab U at p. 544, rt. col., 2nd ¶. The cited "ref. 16" is: Backman, K., Ptashne, M., and Gilbert, W., "Construction of plasmids carrying the cI gene of bacteriophage λ," Proc. Natl. Acad. Sci. USA, (1976) Vol. 73, No. 11, pp. 4174-4178, which recites "E. coli K12 strain 294" (Tab TT at p. 4174, rt. col., Materials and Methods).

77. Claim 31 recites: "A method for producing a first polypeptide having a total of 166 amino acids and having the amino acid sequence of a mature human fibroblast interferon or a second polypeptide having a total of 165 amino acids and having the amino acid sequence of a mature human fibroblast interferon except for the amino-terminal methionine of said interferon or a mixture of said first and second polypeptides, comprising culturing a microorganism transformed with a microbial expression vehicle capable, in said microorganism, of directing expression of a DNA of claim 25 or 26."

a. I have been informed that the 'preamble' of a claim does not impart meaning to the claim where it only states a purpose or intended use of the invention. But regardless, the preamble limitation is disclosed by the prior art. I have been informed when then there are limitations in the alternative, only one must be satisfied. The preamble element "first polypeptide having a total of 166 amino acid and having the amino acid sequence of a mature human fibroblast interferon" and the element "DNA of claim 25 or 26" is disclosed by the Taniguchi Gene paper or the Preprint as discussed above in paragraph 71.

b. The element "comprising culturing a microorganism transformed with a microbial expression vehicle capable, in said microorganism, of directing expression" is disclosed in the Ptashne '892 patent at the same passage cited in paragraph 73 above (see Tab BB at col. 1, ll. 35-48). The passage specifies that a eukaryotic gene can be expressed by a bacterial plasmid having a portable promoter and a hybrid ribosomal binding site, where the plasmid is transformed and cultured in a bacterium.

78. I understand claim 32 differs from claim 31 by limiting the preamble such that the method is only for producing a polypeptide having a total of 166 amino acids and having the amino acid sequence of a mature human fibroblast interferon. As discussed at least in paragraph 77 above, the Ptashne'892 patent provides the method for producing native, unfused proteins and the Taniguchi Gene paper or Preprint provides the cDNA that codes for a polypeptide having a total of 166 amino acids and having the sequence of a mature human fibroblast interferon.

79. I understand claim 33 differs from claim 31 by limiting the preamble such that the method is only for producing a polypeptide having a total of 165 amino acids and having the amino acid sequence of a mature human fibroblast interferon except for the amino-terminal methionine of said interferon. This limitation is disclosed by the common knowledge in the prior art at the time because it was known that N-terminal methionines are sometimes removed by bacterial methionyl aminopeptidases. For example, the Goeddel Nature paper states, "[t]he fact that most bacterial proteins do not contain N-terminal methionine residues suggests that the fMet should be efficiently removed, resulting in the direct expression of HGH." (Tab U, p. 544, lt. col., 2nd to last ¶.) Given this view, bacterial production of a eukaryotic cDNA that codes for a 166 amino acid protein starting with methionine could result in a 165 amino acid protein. With

respect to the culturing step, it is generally the same for a 166 amino acid protein as it would be for a 165 amino acid protein, as it would be for essentially any other protein.

80. Claim 34 recites: "A DNA which consists essentially of a DNA which codes for human fibroblast interferon polypeptide." I have been informed that the broadest reasonable interpretation of the claim would mean that the DNA can code for a human fibroblast interferon polypeptide with or without presequence, especially in view of the fact that claim 35 is identical to this claim except that claim 35 specifies "mature." The Taniguchi Gene paper and the Preprint both disclose cDNA molecules that code for human fibroblast interferon polypeptides, and teach where the leader and mature sequences lie within the cDNA (see Fig. 2 and Fig. 1, respectively). But even if the claim is interpreted narrowly to mean without presequence, at least as of March 19, 1980, cloning techniques necessary to make an interferon cDNA without presequence were well known. As discussed above in paragraph 71, the Ptashne '892 patent informs one how to make an expression plasmid such that the ATG of the gene to be expressed is part of a hybrid ribosomal binding site nearby the portable promoter, and in this manner, transcription and translation are coupled under control of the plasmid. In order to make such an expression plasmid, the Ptashne '892 patent teaches the use of Exonuclease III and S1 to remove unwanted base pairs of the gene. It was well known in the prior art at least as of October 26, 1979[2] or February 2, 1980[3] that the secreted form of human fibroblast interferon began with an

[2] I have reviewed the publication, Knight and Hunkapiller, "Purification, Amino Acid Composition, and N-Terminal Amino Acid Sequence of Human Fibroblast Interferon," from Annals of the NewYork Academy of Sciences, Vol. 350, "Regulatory Functions of Interferons," Eds. Vilcek et al., (1980) (Tab SS). I understand this is a publication of the information presented by Knight and Hunkapiller at the October 23-26, 1979 New York Academy of Sciences Conference on Regulatory Functions of Interferons. The publication discloses the 13 N-terminal amino acids of the secreted mature human fibroblast interferon.

N-terminal sequence of Met-Ser-Tyr-Asn-Leu-Leu-Gly-Phe-Leu-Gln-Arg-Ser-Ser. With this N-terminal sequence and with the cDNA sequence provided by the Taniguchi Gene paper or Taniguchi Preprint, one of ordinary skill in the art would know that the functional, secreted human fibroblast interferon protein did not have the leader sequence. Thus, one of ordinary skill in the art would have made the expression construct such that the leader sequence would not be expressed as part of the interferon protein.

81. Claim 35 recites: "A DNA consisting essentially of a DNA which codes for mature human fibroblast interferon polypeptide having the amino acid sequence: [mature human fibroblast interferon amino acid sequence as shown in claim 35 in Tab JJ]." As discussed above in paragraph 80, the Taniguchi Gene paper and Preprint disclose the nucleotide coding sequence for the mature human fibroblast interferon amino acid sequence because they specify where the mature sequence begins and ends and where the presequence begins and ends. The claimed amino acid sequence is identically disclosed by the Taniguchi disclosures. Also as discussed above in paragraph 71, the Ptashne '892 patent and the prior art (for example, see Goeddel's '832 patent, Tab DD at col. 5, l. 60 to col. 6, l. 15) teaches the use of Exonuclease III and S1 or other nucleases such that "the gene can first be cut at the restriction site, the desired number of base pairs and any single stranded tails can be removed by treating with nucleases for the appropriate time period, and religating." (Tab BB at col. 2, ll. 7-11.)

82. Claim 36 recites: "A DNA consisting essentially of a DNA according to claim 35 which has the base pair sequence: [nucleotide sequence that codes for the mature human fibroblast interferon protein as listed in claim 36 in Tab JJ]." I understand that claim 36 differs from claim 35 by specifying the exact nucleotide sequence that codes for the mature human

[3] The publication date of Knight et al., Science, 207:525-526 (Feb. 1980). I have reviewed this publication, and it also discloses the 13 N-terminal amino acids of the secreted mature human fibroblast interferon.

fibroblast interferon. This nucleotide sequence is disclosed by the Taniguchi Gene paper and the Preprint at Fig. 2 and Fig. 1, respectively. The Taniguchi Gene paper nucleotide sequence at nucleotides 70 to 567 is identical to the nucleotide sequence listed in Goeddel claims 26, 36, and 42, except for the nucleotides that encode amino acid residue 111 (phenylalanine), where the Taniguchi Gene paper recites "TTC" and the Goeddel claims recite "TTT". Because both triplets code for phenylalanine, the single nucleotide difference is inconsequential. The sequences are identical in the Taniguchi Gene paper and in the Taniguchi Preprint.

83. Claim 37 recites: "A cloned DNA consisting essentially of a DNA having the base pair sequence defined in claim 36." I understand that claim 37 differs from claim 36 by specifying that the DNA is "cloned." Figure 2 of the Taniguchi Gene paper provides the sequence from the "cDNA," which means complementary DNA that is synthesized from mRNA by reverse transcriptase. This necessarily means that a cDNA is cloned, which was cloned into "[h]ybrid plasmid TpIF319-13, consists of a cDNA of about 800 base pairs inserted at the EcoRI site of pBR322." (Tab LL at p. 12, lt. col., last ¶.) The Taniguchi Preprint also provides the sequence from the cDNA, and explicitly mentions that "[w]e have recently cloned and sequenced one species each of Le-IF I) (18, 19) and F-IF cDNA (20, 21)." (Tab MM at p. 2, last ¶.) Again, exercising prior art cloning techniques such as those described in the Ptashne '892 patent or in other prior art, one of ordinary skill in the art could routinely make DNA molecules having a sequence that is limited to the coding sequence for the mature human fibroblast interferon by, for example, linearizing the plasmid upstream of the cDNA and digesting back the cDNA with nucleases.

84. Claim 38 recites: "A cloned DNA consisting essentially of a DNA which codes for a polypeptide having the amino acid sequence in claim 35." As discussed in paragraph 81

above, both the Taniguchi Gene and Preprint disclose cloned DNA coding for a polypeptide having the mature human fibroblast interferon amino acid sequence, and the Ptashne '892 patent provides further explicit instructions to make a construct such that a native protein is expressed from its ATG.

85. Claim 39 recites: "A recombinant plasmid wherein a DNA which codes for the amino acid sequence: [mature human fibroblast interferon amino acid sequence as listed in at claim 39 in Tab JJ] is inserted in a vector DNA." I understand that claim 39 differs from claim 38 by the limitation "recombinant plasmid." The Taniguchi Gene paper discloses that the cDNA of human fibroblast interferon was cloned into TpIF319-13, which is a recombinant plasmid. The Taniguchi Preprint discloses that the fibroblast interferon cDNA was cloned and referred to reference number 20 ((Tab NN at p. 2, last ¶), which is Taniguchi et al., Proc. Jap. Acad. Ser. B, 55, 464-469 (1979)(Tab G), which discloses TpIF319-13. (See Tab G at p. 468, 1st full ¶: "These results thus indicate that the recombinant plasmid DNA from clone no. 319, named TIF319, contains the sequence for the interferon mRNA.") The Ptashne '892 patent teaches that for a protein to be expressed in its native, i.e., mature, form, the ATG of the gene is positioned as part of a hybrid ribosomal binding site downstream of a portable promoter. The '892 patent teaches that unwanted basepairs of the gene, which in this case would be at least the ATG of the presequence, are eliminated using Exonuclease III and S1 nuclease.

86. Claim 40 recites: "A microorganism containing the recombinant plasmid defined in claim 39." I understand that claim 40 differs from claim 39 by the limitation "a microorganism" containing the recombinant plasmid of claim 39. The Taniguchi Gene paper states, "a bacterial clone containing the hybrid plasmid TpIF319-13 has been described in previous papers," which previous papers include "Taniguchi et al. 1979," i.e., Taniguchi et al.,

Proc. Jap. Acad. Ser. B, 55, 464-469 (1979)(Tab G). (See Tab LL at p. 12, lt. col., 2nd ¶.) This paper is the same reference number 20 referred to by the Taniguchi Preprint in its disclosure that the fibroblast interferon cDNA was cloned (Tab MM at p. 2, last ¶). A "bacterial clone" is a microorganism containing the recombinant plasmid.

87. Claim 41 recites: "The microorganism according to claim 40 which is Escherichia coli K-12 strain 294." The Goeddel Nature paper discloses K-12 strain 294: "Many transformants of E. coli 294 (ref. 16) were obtained with plasmids containing EcoRI and HindIII inserts of approximately the desired size." (Tab U at p. 544, rt. col., 2nd ¶.) The cited "ref. 16" is: Backman, K., Ptashne, M., and Gilbert, W., "Construction of plasmids carrying the cI gene of bacteriophage λ," Proc. Natl. Acad. Sci. USA, (1976) Vol. 73, No. 11, pp. 4174-4178 (Tab TT), which recites "E. coli K12 strain 294," (Tab TT at p. 4174, rt. col., Materials and Methods.)

88. Claim 42 recites: "The recombinant plasmid according to claim 39 wherein said inserted DNA comprises the following base pair sequence: [nucleotide sequence coding for the mature human fibroblast interferon as shown in claim 42 of Tab JJ]." I understand claim 42 differs from claim 39 by specifying a specific nucleotide sequence coding for the mature human fibroblast interferon amino acid sequence rather than any nucleotide sequence coding for said amino acid sequence. This nucleotide sequence is disclosed by the Taniguchi Gene paper and the Preprint at Fig. 2 and Fig. 1, respectively. The Taniguchi Gene paper nucleotide sequence at nucleotides 70 to 567 is identical to the nucleotide sequence listed in Goeddel claims 26, 36, and 42, except for the nucleotides that encode amino acid residue 111 (phenylalanine), where the Taniguchi Gene paper recites "TTC" and the Goeddel claims recite "TTT". Because both triplets code for phenylalanine, the single nucleotide difference is inconsequential. The sequences are identical in the Taniguchi Gene paper and in the Taniguchi Preprint. Again, both

Taniguchi disclosures are explicit as to where the mature human fibroblast interferon nucleotide and encoded amino acid sequence begins and ends, and the Ptashne '892 patent teaches that unwanted basepairs of the gene, such as the presequence of the interferon leader sequence, can be eliminated using Exonuclease III and S1 nuclease so that translation begins at the desired ATG.

89. Claim 43 recites: "The recombinant plasmid according to claim 39 wherein said vector DNA is an Escherichia coli plasmid." I understand that claim 43 differs from claim 39 by specifying that the vector is an E. coli plasmid. . Both the Taniguchi Gene paper and Preprint disclose the recombinant plasmid TpIF319-13, which is based on the pBR322 plasmid ("Hybrid plasmid TpIF319-13, consists of a cDNA of about 800 base pairs inserted at the EcoRI site of pBR322," see Tab LL at p. 12, lt. col., last ¶), which is an E. coli plasmid. The Taniguchi Preprint discloses TpIF319-13 because it states that the fibroblast interferon cDNA was cloned and referred to reference number 20 (see Tab MM at p. 2, last ¶), which is Taniguchi et al., Proc. Jap. Acad. Ser. B, 55, 464-469 (1979)(Tab G), which discloses TpIF319-13. (See Tab G at p. 468, 1st full ¶: "These results thus indicate that the recombinant plasmid DNA from clone no. 319, named TIF319, contains the sequence for the interferon mRNA.")

90. Claim 44 recites: "The recombinant plasmid according to claim 43 wherein said Escherichia coli plasmid is pBR322." I understand that claim 44 differs from claim 43 by specifying that the plasmid is "pBR322." As discussed above in claim 43, the TpIF319-13 plasmid is made from the pBR322 plasmid.

91. Claim 45 recites: "A process for preparing a recombinant plasmid which comprises inserting a synthesized double stranded DNA which codes for the amino acid

sequence: [mature fibroblast human interferon amino acid sequence as listed in claim 45 in Tab JJ] in a vector DNA."

a. The step of "inserting a synthesized double stranded DNA" is disclosed by the Taniguchi Gene reference by its statement that "DNA synthesized by in vitro reverse transcription of the interferon mRNA has been cloned and amplified as recombinant DNA, TpIF319-13 (Taniguchi et al. 1979)." (Tab LL at Abstract, emphasis added.) Reverse transcription of interferon mRNA will result in a "synthesized double stranded DNA" which is inserted into a vector DNA because cloning it into TpIF319-13 means it is inserted into a vector DNA. The Taniguchi Preprint also discloses the step "inserting a synthesized double stranded DNA" into "a vector DNA" because the Preprint mentions that "[w]e have recently cloned and sequenced one species each of Le-IF I) (18, 19) and F-IF cDNA (20, 21)." (Tab MM at p. 2, last ¶.) Cloning the F-IF cDNA in reference 20 involved synthesizing cDNA from interferon mRNA and inserting the synthesized cDNA into pBR322 to generate TpIF319-13.

b. The synthesized double stranded DNA that codes for the mature human fibroblast interferon amino acid sequence is disclosed in the prior art because Fig. 2 of the Taniguchi Gene paper and Fig. 1 of the Preprint present the interferon cDNA sequence and encoded mature amino acid sequence, where amino acids 1-166 are identical to the claimed amino acid sequence. Further, the '892 Ptashne patent teaches how to express eukaryotic proteins in bacteria by constructing expression vectors where a gene that codes for the native eukaryotic protein is inserted such that translation begins at its ATG.

92. Claim 46 recites: "The process according to claim 45 wherein said vector DNA is an Escherichia coli plasmid." I understand that claim 46 differs from claim 45 by specifying that the vector DNA is an E. coli plasmid. Both the Taniguchi Gene paper and the Preprint disclose

that the vector DNA is an E. coli plasmid because both publications disclose that the interferon cDNA was cloned in TpIF319-13, a pBR322 based E. coli plasmid.

93. Claim 47 recites: "A process according to claim 46 wherein said Escherichia coli plasmid is pBR322." I understand that claim 47 differs from claim 46 by specifying that the E. coli plasmid is pBR322. As stated in paragraph 69 above, TpIF319-13 is a pBR322 based plasmid.

94. Claim 48 recites: "A process for producing a microorganism capable of expression of a polypeptide with interferon activity which comprises transforming a host microorganism with a replicable recombinant plasmid containing a foreign DNA which codes for the amino acid sequence: [mature human fibroblast interferon amino acid sequence as listed in claim 48 of Tab JJ]. The Ptashne '892 patent discloses a method for producing a microorganism with a replicable plasmid capable of expressing a polypeptide with interferon activity:

> The present invention provides a method of producing native, unfused prokaryotic or eukaryotic protein in bacteria which comprises inserting into a bacterial plasmid a gene for a prokaryotic or eukaryotic protein and a portable promoter consisting of a DNA fragment containing a transcription initiation site recognized by RNA polymerase and containing no protein translational start site, said promoter being inserted ahead of a protein translational start site of said gene to form a fused gene having a hybrid ribosome binding site, inserting said plasmid into said bacteria to transform said bacteria with said plasmid containing said fused gene, and culturing the transformed bacteria to produce said prokaryotic or eukaryotic protein."

(Tab BB at col. 1, ll. 35-48.) As recited in this passage, the Ptashne '892 patent teaches how to make an expression vector that can direct the expression of native proteins, and that this expression vector is transformed into bacteria for production. As emphasized in the Abstract, "[t]he invention, which uses recombinant DNA techniques, produces proteins in their natural, functional state unencumbered by extraneous peptides." In reading the Ptashne '892 patent combined with the sequence information provided by the Taniguchi Gene paper or Preprint, one

of ordinary skill in the art would know to make a hybrid ribosomal binding site with the mature interferon ATG, such that the "extraneous peptides" of the leader sequence would not be present. Again, the Taniguchi Gene paper or Preprint provide (residues 1-166) the claimed amino acid sequence for the mature human fibroblast interferon.

95. Claim 49 recites: "A process according to claim 48 wherein said host microorganism is Escherichia coli K-12 strain 294." As discussed above in paragraph 87, the limitation "Escherichia coli K-12 strain 294" is disclosed in the Goeddel Nature paper.

96. Claim 50 recites: "A recombinant expression plasmid, comprising a DNA sequence encoding a mature human fibroblast interferon having a total of 166 amino acids and unaccompanied by a human fibroblast interferon presequence operably linked therein for expression by an Escherichia coli host, whereby expression of a mature human fibroblast interferon having a total of 166 amino acids and unaccompanied by a human fibroblast interferon presequence by said host is enabled." The Ptashne '892 patent teaches that expression of a native protein without extraneous peptides is made by fusing the ATG of the native protein to make a hybrid ribosomal binding site, "a hybrid ribosomal binding site is formed consisting of the S-D sequence and initiator from the lac operon and the ATG sequence of the gene, and such a fused gene is translated and transcribed efficiently." (Tab BB at col. 1, l. 66 to col. 2, l. 2.) The Taniguchi Gene paper and Preprint teach where the presequence ends and where the ATG of the mature human fibroblast begins. The Ptashne '892 patent teaches operably linked expression is governed by a promoter and a hybrid ribosomal binding site:

> [T]he *E. coli lac* operon, a fragment of DNA which contains a transcription initiation site but no translational start site, has the required properties to function as a portable promoter in the present invention, being transcribed at high efficiency by bacterial RNA polymerase. The mRNA produced contains a Shine-Dalgarno (S-D) Sequence but it does not include the AUG or GUG required for translational initiation. However, in accordance with the present invention, a

> hybrid ribosomal binding site is formed consisting of the S-D sequence and initiator from the lac operon and the ATG sequence of the gene, and such a fused gene is translated and transcribed efficiently.

(Tab BB at col. 1, l. 57 to col. 2, l. 2.) The Ptashne '892 patent further teaches how unnecessary base pairs of the gene can be removed, "[s]ince the promoter can be inserted at a restriction site ahead of the translational start site of the gene, the gene can first be cut at the restriction site, the desired number of base pairs and any single stranded tails can be removed by treating with nucleases for the appropriate time period, and religating." (Tab BB at col. 2, ll. 5-11.) Thus, given the knowledge of the human fibroblast interferon presequence provided by the Taniguchi Gene paper or Preprint, the Ptashne '892 patent teaches one of ordinary skill in the art that the presequence can be digested away with nucleases. A variety of timed digestions can be performed to remove different lengths of the presequence (where timed digestions was common practice in the art as of March 19, 1980), including the whole presequence, but digestion of the presequence ATG is all that is required in order to ensure translation begins from the mature interferon ATG such that the produced protein is unaccompanied by presequence.

97. Claim 51 recites: "An Escherichia coli host cell capable of expressing a mature human fibroblast interferon polypeptide having a total of 166 amino acids and unaccompanied by a human fibroblast interferon presequence, transformed with an expression plasmid of claim 50." The Ptashne '892 patent teaches that E. coli is capable of expressing eukaryotic proteins from expression vectors designed according to its methods as described above. As stated in claim 2 of the Ptashne '892 patent, "The method as claimed in claim 1 in which said bacteria is *E. coli*." (Tab BB at col. 3, ll. 19-20.)

98. Claim 52 recites: "A method of producing a mature human fibroblast interferon polypeptide having a total of 166 amino acids and unaccompanied by a human fibroblast interferon presequence, comprising expressing the expression plasmid of claim 50 in Escherichia

coli." As discussed in paragraph 96 above, the Ptashne '892 patent teaches that expression in bacteria is controlled by the plasmid's portable promoter and hybrid ribosomal binding site, which are operably linked to the gene to be expressed. Thus, the Ptashne '892 patent teaches "expressing the expression plasmid of claim 50 in Escherichia coli."

C. Goeddel U.S. Patent No. 5,460,811 ('811 patent) claims 1-6 are made obvious by the Taniguchi Preprint or the Taniguchi Gene paper in view of the Ptashne '892 patent, Anonymous Research Disclosure, and optionally with Fujisawa:

99. I have reviewed the '811 patent (Tab V) and its claims (Tab KK) involved in the interference. Claim 1 recites: "A composition comprising water and a nonglycosylated polypeptide having the amino acid sequence of a mature human fibroblast interferon, said nonglycosylated polypeptide having a total of 165 or 166 amino acids and said composition being free of any glycosylated human fibroblast interferon."

a. The Taniguchi Gene paper or Preprint disclose a cDNA that codes for the mature human fibroblast interferon having 166 amino acids, which amino acid sequence is disclosed. (See residues 1-166 in Tab LL at Fig. 2 and its legend, or in Tab MM at Fig. 1 and its legend.) The Ptashne '892 patent teaches how to express a gene to produce a native, functional, unfused protein by operably linking a *lac* promoter to a hybrid ribosomal binding site such that translation begins at the gene's ATG. (See for example paragraphs 33, 46, 71, and 96.) The Ptashne '892 patent also teaches how to remove unnecessary base pairs, such as a leader sequence, so that the ATG is properly positioned with respect to the promoter and the Shine-Delgarno sequence (see paragraphs 44-46). Thus, in my opinion, one of ordinary skill in the art having the combined disclosures of the Taniguchi Gene paper or the Taniguchi Preprint and the Ptashne '892 patent would have been able as of May 19, 1980 (with the Taniguchi Gene paper) or as of April 8, 1980 (with the Taniguchi Preprint) to construct without undue experimentation

an expression construct that produces in bacteria a human fibroblast protein having 166 amino acids without presequence (*i.e.,* a mature human fibroblast protein).

b. A bacterial protein extract containing expressed human fibroblast interferon would necessarily contain water because cellular mixtures are not anhydrous. It is common knowledge that bacteria are water-based organisms.

c. Further, it is a total certainty that an interferon produced in E. coli would be completely non-glycosylated. An interferon produced in E. coli is necessarily completely, 100% non-glycosylated because the only known bacterial strain that performs N-linked glycosylation is *Campylobacter jejuni.* For example, the paper "Glycosylation in Bacteria," Science, 298, 1675, 1677 (2002), (Tab VV), states at 1677: "N-linked glycosylation is a common posttranslational modification of membrane and secretory proteins in eukaryotes. However, the only bacterium known to modify proteins in this way is *Campylobacter jejuni*, and thus many eukaryotic proteins generated in bacterial systems are of limited use because they lack appropriate modifications."

d. In addition, the Fujisawa paper reports that non-glycosylated mouse L cell interferon is functional. The investigators used tunicamycin, a specific inhibitor of protein glycosylation, in order to completely or substantially remove carbohydrate modifications on interferon. As the authors state, these experiments were conducted to address the issue that prior experiments may not have completely removed the carbohydrates:

> Dispensability of at least part of the carbohydrate moiety for the antiviral activity was indicated by glycosidase treatment of human interferons (3). Molecular heterogeneity of some interferons appears to be due to variations in their carbohydrate moieties. Removal of the carbohydrate by treatment of interferon with glycosidases or periodate, or by inhibition of glycosylation during its biosynthesis, resulted in reduced charge heterogeneity, as well as in decreased molecular weights (3-5). But these methods may not completely remove the carbohydrate, or they may entail inactivation and some side effects on the

> molecule. To clarify the role of the carbohydrate moiety of interferon, we have examined the effect of tunicamycin on production of mouse L cell interferon induced by Newcastle disease virus. This antibiotic specifically inhibits formation of the *N*-acetyl-glucosamine-lipid intermediate involved in *N*-glycosidic linking of core oligosaccharides to protein (6-8), and has been successfully used to inhibit glycosylation of various secretory and viral membrane proteins (9-14). ... In this communication, we report that inhibition of glycosylation by tunicamycin eliminates interferon molecules of normal size and forces molecules of a smaller size containing little or no sugar but with full antiviral activity to appear.

(Tab PP at p. 8677, lt. col., 2nd ¶ to rt. col., 1st ¶.) Elsewhere in the paper, the authors again emphasize that the use of tunicamycin results in some fractions where interferon was completely non-glycosylated, "It is concluded that the small interferon molecules (T) produced in the presence of tunicamycin contain little or no carbohydrate." (Id. at p. 8678, lt. col., 3rd ¶.) They conclude that "tunicamycin appears to be a useful agent, in its specificity and completeness of action, to produce nonglycosylated and fully active interferon molecules." (Id. at p. 8678, lt. col., 3rd ¶.) Thus, in my opinion, one of ordinary skill in the art, having read the Fujisawa paper, would have found it likely that completely non-glycosylated human interferons would maintain anti-viral activity.

100. With respect to the scope and content of the prior art regarding non-glycosylated interferons, I have also reviewed the following publications:

Havell et al., "Suppression of Human Interferon Production by Inhibitors of Glycosylation," Virology, 63, pp. 475-483 (1975) (Tab WW; "Havell (1975)");

Bose et al., "Apparent Dispensability of the Carbohydrate Moiety of Human Interferon for Antiviral Activity," J. Biol. Chem., Vol. 251, No. 6, pp. 1659-1662 (1976) (Tab XX); and

Havell et al., "Altered Molecular Species of Human Interferon Produced in the Presence of Inhibitors of Glycosylation," J. Biol. Chem., Vol. 252, No. 12, pp. 4425-4427 (1977) (Tab YY; "Havell (1977)").

101. The above-listed papers in paragraph 27 indicate that the carbohydrate modifications on interferons are likely not necessary for function. In fact, a similar statement was made in the Havell (1975) paper: "It remains to be explained why proper glycosylation may be required for the synthesis of biologically active interferon. Although it seems attractive to speculate that the carbohydrate portion is important in the interaction of interferon with a cellular receptor site on target cells, this interpretation appears unlikely in view of two findings: it was shown that rabbit interferon retains full biological activity after removal sialic acid (Schonne et al., 1970; Dorner et al. 1973), and enzymatic removal of about 50% of total carbohydrate from a preparation of human interferon also failed to reduce biological activity (C.B. Anfinsen, personal communication)." (Tab WW at p. 482.) The Bose paper showed that removal of about 80% of sugar moieties on human interferons did not affect function. (Tab XX at Abstract.) As discussed above, the Fujisawa paper experiments were conducted to address the issue that prior experiments may not have completely removed the carbohydrates, so that they reported the "inhibition of glycosylation by tunicamycin eliminates interferon molecules of normal size and forces molecules of a smaller size containing little or no sugar but with full antiviral activity to appear." (Tab at p. 8677, rt. col., 1st ¶.) Thus, one or ordinary skill as of March 19, 1980 attempting to express interferon in bacteria had a basis to believe that a completely non-glycosylated interferon would maintain function.

100. Claim 2 recites: "The composition of claim 1, said nonglycosylated polypeptide having the amino acid sequence [as shown in claim 2 of Tab KK; the mature human fibroblast interferon sequence, except that residue 1 can be H or Met]." I understand that claim 2 limits claim 1 by specifying the particular amino acid sequence. The Taniguchi Gene paper or Preprint discloses the amino acid sequence of claim 2 where residue 1 is Methionine, *i.e.,* amino acid

residues 1-166 shown in Fig. 2 and Fig. 1, respectively. Given the Ptashne '892 patent and either the Taniguchi Gene paper or Preprint, one of ordinary skill in the art would know how to make a non-glycosylated polypeptide having the amino acid sequence of the mature human fibroblast interferon because: (1) the Ptashne '892 patent teaches a method for expressing a native protein by positioning its ATG downstream of a portable promoter, as part of a hybrid ribosomal binding site, (2) the Ptashne '892 patent teaches how to remove unnecessary base pairs of the gene to be expressed, and (3) the Taniguchi Gene paper and Preprint disclose where the leader sequence ends and where the ATG of the mature interferon begins. (See paragraphs 33, 46, 56-57, 71, and 96.) Thus, in order to express the mature interferon protein as disclosed by the Taniguchi disclosures and with the method of the Ptashne' 892 patent, one of ordinary skill would know to clone the cDNA of interferon into a plasmid and digest away at least the unnecessary ATG of the leader sequence, such that the mature interferon ATG is fused to the bacterial Shine-Delgarno sequence and positioned downstream of the *lac* promoter.

101. Claim 3 recites: "The composition of claim 2, said nonglycosylated polypeptide having a formula molecular weight of about 20,027." I understand that claim 3 differs from claim 2 by specifying that the polypeptide has a molecular weight of 20,027.

a. It would have been common knowledge in the prior art as of April 8, 1980[4] (with respect to the Taniguchi Preprint) or as of May 19, 1980[5] (with respect to the Taniguchi Gene paper) to predict that a polypeptide expressed in bacteria having the amino acid sequence of the mature human fibroblast protein would have a molecular weight of about 20 kilodaltons.

[4] I have been informed that the Taniguchi Preprint was distributed to the public in late March 1980, and received by individuals at least by April 8, 1980.

[5] I have been informed that the Taniguchi Gene paper was distributed to the public on May 19, 1980, and was received and indexed in a library at least by June 2, 1980.

Because proteins expressed in E. coli are necessarily unglycosylated, the molecular weight of the protein is based on the molecular weight of its amino acid composition. Well before 1980, it was common knowledge for one of ordinary skill to compute the molecular weight of a protein based on its primary amino acid sequence. Thus, a "nonglycosylated polypeptide having a formula molecular weight of about 20,027" is disclosed by the combination of the Taniguchi Gene paper or Preprint mature human fibroblast amino acid sequence (residues 1-166) in view of common knowledge of the prior art.

b. Alternatively, a protein produced in bacteria from the cDNA coding for the mature human fibroblast interferon would necessarily have a molecular weight according to its amino acid composition. For example, the Taniguchi et al., Proc. Natl. Acad. Sci. USA, Vol. 77, No. 9, pp. 5230-5233 (Sept. 1980) (Tab ZZ) reported the bacterial expression of human fibroblast using the Ptashne lab method ("We applied the method of Guarente et al. ... [(1980) *Cell* 20, 543-553] to construct plasmids that direct expression in *Escherichia coli* of the human fibroblast interferon (F-IF) gene." (See Tab ZZ at Abstract)). The interferon produced had a molecular weight of about 20 kilodaltons:

> These plasmids bear a *lac* portable promoter abutted to the ATG encoding the amino-terminal methionine of pre-F-IF (pLG104R) and to the ATG encoding the amino-terminal methionine of F-IF (pLG117R). [....] When compared with pBR322, pLG117R and pLG104R each direct synthesis of one new protein of molecular weights approximately 20,000 and 23,000 respectively. These are the sizes expected for unglycosylated proteins with the primary sequences of F-IF and pre-F-IF as predicted from the DNA sequence of Taniguchi et al. (6) [i.e., the Taniguchi Gene paper].

(Tab ZZ at p. 5233, lt. col., 1st ¶.) Thus, a "nonglycosylated polypeptide having a formula molecular weight of about 20,027" is necessarily disclosed by the production of a mature human fibroblast interferon protein in bacteria.

102. Claim 4 recites: "The composition of claim 1, 2 or 3, said composition being free of human proteins." This limitation is necessarily disclosed by the combined disclosure of the Taniguchi Gene paper or Preprint in view of the Ptashne '892 patent because proteins produced in bacteria are necessarily free of (other) human proteins.

103. Claim 5 recites: "The composition of claim 1, 2 or 3, said composition containing a therapeutically effective amount of said nonglycosylated polypeptide and being suitable for parenteral administration." The Anonymous Research Disclosure implicitly discloses the claim 5 limitations because the publication refers to the clinical application of human interferon:

> Since the discovery of interferon in 1957[1], a number of attempts have been made to demonstrate the effectiveness of the product in the clinic. Initially, interest focused around its use as an anti-viral compound but when it was realised that its antiviral action was not its only effect on cells interest turned to interferon as an anti-cancer agent. Material is now being used in clinical trials against both viral diseases[3] and cancer[4] and it is not inconceivable that it will also be used in the future as a modulator of the immune system[5] eg in tissue graft situations. [....] The problem of shortage of human interferon for clinical studies is still the major drawback. [....] However, probably the best way to produce large quantities of interferon will be to introduce the gene(s) for interferon production into bacteria.[11]

(Tab D at 1st pg., 1st ¶ to 2nd ¶.)

a. Because the passage informs the reader that the "best way" to produce interferon is by bacterial production, the limitation "nonglycosylated" is inherently disclosed. The limitation of nonglycosylated interferon is also inherently disclosed by the combined disclosures of the Taniguchi Gene paper or Preprint and the Ptashne '892 patent, as the combined disclosures provide for the production of interferon in bacteria.

b. Reading the above passage, one of ordinary skill in the art as of March 19, 1980 would have known to look to the teachings from the publications cited in the phrase "clinical trials against both viral diseases[3] and cancer[4]" in order to what a therapeutically effective amount of interferon might be for parenteral administration. The cited publication "3" is Greenberg et

al., "Effect of Human Leukocyte Interferon on Hepatitis B Virus Infection in Patients with Chronic Active Hepatitis," N. Engl. J. Med., Vol. 295, No. 10, pp. 517-522 (Sept. 1976) (Tab B). The Abstract of this reference teaches a therapeutically effective amount of interferon for parenteral administration:

> Four patients with chronic hepatitis B infection and chronic active hepatitis were treated with human leukocyte interferon. Three of them had consistently elevated levels of circulating Dane-particle markers, including Dane-particle-associated DNA polymerase activity, hepatitis B core antigen and Dane-particle-associated DNA. Parenteral interferon administration at a dosage between 6.0×10^3 and 17×10^4 U per kilogram per day was associated with rapid and reproducible fall in all Dane-particle markers in the three patients.

(Tab B at Abstract.) The passage explicitly recites "parenteral interferon administration," and it provides a dosage that was therapeutically effective as levels of "all Dane-particle markers" (which indicates a decrease in virus in the patient) fell in the patients. Although the interferon administered in the Greenberg reference was leukocyte interferon, one of ordinary skill in the art would have attempted the disclosed dosages for fibroblast interferon because it was well-established at the time that fibroblast interferon had antiviral capabilities.

104. Claim 6 recites: "The composition of claim 4, said composition containing a therapeutically effective amount of said nonglycosylated polypeptide and being suitable for parenteral administration." I understand that claim 6 differs from claim 5 by having the additional limitation that the interferon to be administered is "free of human proteins." This limitation is necessarily disclosed by a teaching of interferon production in bacteria because any interferon purified from bacteria is necessarily free of (other) human proteins. The combined disclosures of the Taniguchi Gene paper or Preprint and the Ptashne '892 patent provide for the teaching of interferon production in bacteria, as does the Anonymous Research Disclosure passage discussed above in paragraph 103.

The Japanese Application 33931/80 is an Enabling Disclosure for a Cloned cDNA Consisting of the Mature Human Fibroblast Interferon Sequence and for Making the Mature Human Fibroblast Interferon in Bacteria

105. I have reviewed the English translation of Japanese Patent Application No. 55-33931/80 filed on March 19, 1980 (Tab NN; hereafter referred to as the " '931 application"). Page 15 of this application lists the complete nucleotide sequence of the human fibroblast interferon cDNA and encoded amino acids. The complete protein sequence of human fibroblast interferon is also listed because the amino acid sequence contains the leader or presequence of interferon as well as the mature protein sequence. Although the sequence diagram itself does not annotate where the leader sequence ends and where the mature protein sequence begins, any person of skill in the art at the time would have immediately understood the location of the N-terminal start point of the mature interferon sequence. This location would have been immediately known to one of skill in the art because the application also refers to the Knight publication which discloses the first 13 amino acid residues that make up the N-terminal sequence of mature human interferon beta (Knight et al., "Human Fibroblast Interferon: Amino Acid Analysis and Amino Terminal Amino Acid Sequence," Science, vol. 207, pp. 525-526 (1980)(Tab OO)). Figure 3 of the Knight article discloses the N-terminal 13 amino acids of the mature functional human fibroblast interferon protein. This would have been easily matched to the sequence shown on page 15 of the Japanese application.

106. In particular, it would have been immediately clear to a person of ordinary skill in the art that the presequence consists of the first 21 amino acids because the Knight disclosure teaches that the mature sequence begins with the amino acid sequence Met-Ser-Tyr-Asn-Leu-Leu-Gly-Phe-Leu-Gln-Arg-Ser-Ser (see Tab OO at Fig. 3), which corresponds to amino acids 22-34 listed at page 15 of the Japanese Patent Application (see Tab NN at page 15). In fact, the Japanese Patent Application explicitly cites the Knight Science reference at page 16: "It is

important that in the sequence there exist without any errors the base sequence [three base pairs] corresponding to the amino acid sequence from the amino-terminal to the 13th amino acid of the human fibroblast interferon reported by Knight, et al. [Science vol. 207, p. 525-526, (1980)]." (See Tab NN at p. 16.)

The Taniguchi Preprint is an Enabling Disclosure for a Cloned cDNA Consisting of the Mature Human Fibroblast Interferon Sequence and for Making the Mature Human Fibroblast Interferon in Bacteria

107. As discussed herein, the Taniguchi Preprint contains the complete cDNA sequence of human fibroblast interferon, including demarcations of where the leader sequence begins and ends and where the coding sequence for the mature human fibroblast interferon protein begins and ends. Above, I have given my opinion that the Japanese Patent Application No. 55-33931/80, filed March 19, 1980, is an "enabling disclosure" for making a human fibroblast interferon protein in bacteria because the prior art as of March 19, 1980 provided detailed teachings (*i.e.*, the Ptashne Lab papers) regarding how to make a protein in bacteria in its native, unfused form. Likewise, in my opinion, one of ordinary skill in the art, given the Taniguchi Preprint, would have been able to make a human fibroblast interferon protein in bacteria using only the teachings of the prior art. As discussed herein, the Ptashne lab papers as of March 19, 1980 provide explicit details and suggestions as how to make any eukaryotic protein in its native, functional, unfused form, in bacteria.

108. I reserve the right to revise, supplement, and amend this declaration.

109. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements so made are punishable by fine or imprisonment or both under § 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity or enforceability of any patent or patent application surviving this interference.

DATED: 2/13/07 BY: _____

THOMAS M. ROBERTS, PH.D.

TAB A

Date Prepared: 1/18/2007

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Name: Thomas McCoy Roberts

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1976 Ph.D.	Harvard University, Cambridge, Massachusetts

Postdoctoral Training:

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Research Fellowships:

1972-1975	NSF Predoctoral Fellow at Harvard University
1977-1979	NIH Postdoctoral Fellow at Harvard University

Academic Appointments:

1979-1980	Research Associate, Harvard University, Cambridge, MA
1980-1986	Assistant Professor of Pathology, Harvard Medical School and Dana-Farber Cancer Institute, Boston, MA
1986-1990	Associate Professor of Pathology, Harvard Medical School and Dana-Farber Cancer Institute, Boston, MA
1990-	Professor of Pathology, Harvard Medical School and Dana-Farber Cancer Institute, Boston, MA
1995-	Chair, Division of Medical Sciences, Harvard Medical School
1995-	Faculty Dean for Graduate Education, Harvard Medical School

Awards and Honors:

1969	Phi Beta Kappa, Wabash College
1970	A.B., Summa Cum Laude, Wabash College
2002	Honorary Ph.D Wabash college
2003	Honorary Advisor to the Governor of Indiana

Memberships:

Sigma Xi
American Association for the Advancement of Science
American Society for Microbiology

Major Research Interests:

Molecular Analysis of Signal Transduction in Cancer

Administrative Responsibilities:

Chair: Department of Cancer Biology, Dana Farber Cancer Institute

Co-director for the Dana Farber/Novartis Drug discovery program(Yearly budget~$9 million spread among roughly 20 laboratories)

Member: Executive Committee for Research, Dana Farber Cancer Institute

Teaching Experience:

1974-1975	Teaching Assistant (Biochemistry), Harvard University
1977	Teaching Fellow, Marine Biological Laboratories, Woods Hole, MA
1981-1982	Undergraduate Thesis Advisor, Biochemistry 99, Harvard University
1981-	Currently overseeing the thesis and fellowships work of 3 graduate students and 10 postdoctoral fellows
1987-1989	Course Co-Director, Molecular Cloning of Eukaryotic Genes, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
1987	Course Co-Director, Biochemistry 165: Oncogenes: Structure and Function, Department of Biochemistry and Molecular Biology, Harvard University, MA

1994- Course Co-Director, Cell Biology 211b Animal Cell Growth Factors

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3. Roberts, T. M., and K. E. Koths. 1976. The blue-green alga agmenellum quadruplicatum contains covalently closed DNA circles. *Cell 9:551.*

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TAB B

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EFFECT OF HUMAN LEUKOCYTE INTERFERON ON HEPATITIS B VIRUS INFECTION IN PATIENTS WITH CHRONIC ACTIVE HEPATITIS

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Abstract Four patients with chronic hepatitis B infection and chronic active hepatitis were treated with human leukocyte interferon. Three of them had consistently elevated levels of circulating Dane-particle markers, including Dane-particle-associated DNA polymerase activity, hepatitis B core antigen and Dane-particle-associated DNA. Parenteral interferon administration at a dosage between 6.0×10^3 and 17×10^4 U per kilogram per day was associated with a rapid and reproducible fall in all Dane-particle markers in the three patients. The suppressive effect was transient when the interferon was given for 10 days or less but appeared to be more permanent when administration was prolonged for a month or more. In addition, long-term interferon therapy was associated with a marked fall in hepatitis B surface antigen in two of three patients and a disappearance of e antigen in two of two patients. Interferon may be useful in limiting carrier infectivity or eradicating chronic infection. (N Engl J Med 295:517-522, 1976)

CHRONIC infection with hepatitis B virus is manifested by persistence of hepatitis B surface antigen (HB_s Ag) in the blood. In this country approximately 10 per cent of patients hospitalized with acute hepatitis B become chronically infected.[1] Between 0.05 and 0.5 per cent of blood donors in this country and as many as 5 to 15 per cent of the population in some underdeveloped countries have been shown to be HB_s Ag positive at any one time.[2] It has been estimated that 100 million people in the world today are chronically infected with hepatitis B virus.[3] Most chronic infections with this virus persist for many years,[1,4] and spontaneous resolution of the HB_s Ag carrier state has been found to occur only infrequently.[1]

The medical importance of chronic infection with hepatitis B virus results from associated disease, such as chronic active hepatitis in infected patients and the hazard that such patients represent for spread of infection to contacts. Chronic HB_s Ag carriers transmit infection by blood transfusion[2] and by nontransfusion-associated routes.[5-10] The large number of chronic carriers worldwide and the common occurrence of neonatal transmission from mothers who are chronic carriers[9,10] suggest that elimination of this virus from the population by vaccination is unlikely in the foreseeable future. At present no form of therapy has been shown to affect the course of chronic hepatitis B virus infection.

Interferon, a naturally occurring glycoprotein with a wide spectrum of in vitro and in vivo antiviral effects, has been evaluated as a treatment for several human viral infections.[11] The studies described here evaluate the effect of exogenous human leukocyte interferon on chronic hepatitis B virus infection associated with chronic active hepatitis.

Several forms of HB_s Ag have been found in the blood. The most numerous are 20-nm spherical particles and filamentous forms that are free of nucleic acid[12] and represent incomplete virus coat particles. More complex, larger-diameter structures called Dane particles[13] contain HB_s Ag on their surface, a unique internal core antigen (HB_c Ag),[14] a DNA-dependent DNA polymerase[15,16] and small, circular double-stranded DNA.[17] Dane particles are thought to represent the complete hepatitis B virus.

Patients with consistently high levels of Dane-particle-associated DNA polymerase were chosen for this study to determine the effects of interferon on the specific Dane-particle markers (HB_c Ag, Dane-particle DNA polymerase and Dane-particle DNA) as

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well as on total HB_s Ag. e antigen has also been followed. It is distinct from HB_s Ag, but associated with hepatitis B virus infection and with maternal-infant transmission.[10]

Materials and Methods

The four patients selected for study were HB_s Ag carriers for more than six months, had persistently abnormal results on liver-function tests, and had liver biopsies consistent with chronic active hepatitis. In addition, the first three patients were chosen because they had consistently elevated levels of Dane-particle-associated DNA polymerase in their serum. Informed consent was obtained from all subjects.

Serum HB_s Ag was measured by Ausria II (Abbott Laboratories, Chicago, Illinois) and by complement fixation, using a guinea pig antiserum.[18] Anti-HB_s was measured by passive hemagglutination.[19] A solid-phase radioimmunoassay was used to detect serum hepatitis B core antigen (HB_c Ag).[20] Dane particles in 400 μl of serum were pelleted through a 10/20/30 per cent discontinuous sucrose gradient in an SW 40 rotor × 200,000 g for four hours. Pelleted Dane particles were disrupted with 0.5 per cent Nonidet P-40 (Shell Chemicals Limited, London, England) and assayed for HB_c Ag activity, which was expressed as the ratio of counts per minute in the specimen to those found in a negative control. Anti-HB_c was measured by a microtiter adoption of the radioimmunoassay of Robinson.[21] Dane-particle-associated DNA concentrations in serum were measured by a hybridization technic (Lutwick LI, Robinson WS: unpublished data). In brief, Dane particles in 1 to 2 ml of serum were pelleted through 10/20/30 per cent discontinuous sucrose gradient in an SW 40 rotor (× 200,000 g for four hours). The DNA in the pellet was isolated by digestion in 1 per cent sodium dodecyl sulfate and pronase (1 mg per milliliter), followed by phenol extraction and alcohol precipitation. The amount of Dane-particle DNA was determined by measurement of its effect on the reassociation half-time of a high-specific-activity[32] P-DNA made radioactive in a Dane-particle DNA polymerase reaction. A standard curve of reassociation half-time for known concentrations of Dane-particle DNA was used to calculate the Dane-particle DNA concentration in the unknown specimen. Dane-particle-associated DNA polymerase activity, expressed as picomoles of deoxynucleotide triphosphate (dNTP) incorporated into an acid-insoluble form per hour per milliliter by the Dane particles pelleted from 200 or 400 μl of serum, was measured by the method of Robinson.[21] Polymerase activity was shown to be associated with the Dane particles by a specific immune precipitation test employing anti-HB_s and anti-HB_c containing serum[21] or by the demonstration that the radiolabeled product of the DNA polymerase reaction had the same sedimentation coefficient as the product of a control Dane-particle DNA polymerase reaction.[15] e antigen and antibody were measured by gel diffusion. Control antigens and antibodies gave lines of identity with reagents kindly supplied by Dr. James Maynard (Center for Disease Control, Phoenix, Arizona). Serum specimens were concentrated approximately three-fold by ultrafiltration (Amicon, Lexington, Massachusetts) before testing. Interferon was supplied by the Antivirals Substance Program of the National Institute of Allergy and Infectious Diseases. It was used in this study under an Investigational New Drug permit administered by the Bureau of Biologics of the United States Food and Drug Administration and was given subcutaneously once a day (except in Case 1, course A and B, when it was given every 12 hours by intramuscular injection). The interferon was produced by Sendai-virus stimulation of human-blood buffy coats at the Red Cross Blood Transfusion Center in Helsinki, Finland, by Dr. Kari Cantell as described in detail elsewhere.[22] The specific activity of this preparation was 5×10^5 units per milligram of protein.

Serum interferon levels were measured in a plaque-inhibition assay.[23] Interferon unitage was expressed in terms of reference standard 69/19 supplied by the National Institute of Allergy and Infectious Diseases, Bethesda, Maryland. Complete blood counts, serum glutamic oxalacetic transaminase, serum glutamic pyruvic transaminase, alkaline phosphatase and bilirubin were measured weekly in the routine clinical chemistry laboratory.

Results

Subjects

Twenty-five HB_s Ag-positive patients with biopsy-documented chronic active hepatitis were tested for serum Dane-particle-associated DNA polymerase, and seven (28 per cent) had elevated levels. Three of these seven (Case 1, 21 years of age, Case 2, 32, and Case 3, 30) were treated with interferon. In addition, one patient (Case 4, 40 years of age) without detectable DNA polymerase was given interferon.

Abnormal liver function and HB_s Ag were known to have been present for at least six months in all and as long as four years in Case 2. Histologic findings ranged from the earliest changes of chronic active hepatitis (Case 1) to well established cirrhosis (Case 2). Erosion of the limiting plate ("piecemeal necrosis") was mild to moderate in all patients. Two patients had been treated with prednisone before the trial (Cases 2 and 3); one (Case 2) remained on 30 mg of prednisone daily during the study period. All patients were asymptomatic at the time of study, and none had ever had signs or symptoms of severe hepatic decompensation.

Virologic Measurements

In Cases 1, 2 and 3, Dane-particle-associated DNA polymerase levels were relatively constant over the two months before study (Fig. 1-3). Four additional chronically infected patients with elevated polymerase levels had four polymerase determinations over a period of three to 12 months. In these patients, the standard deviation varied less than 20 per cent of the mean.

Parenteral interferon given in a dosage range between 17×10^4 and 6.0×10^3 U per kilogram per day was associated with an immediate and reproducible fall in DNA polymerase levels. In Case 1 (Fig. 1), three successive courses of interferon were all associated with a 10-fold or greater decrease in polymerase levels. In Case 2 (Fig. 2), interferon dosage was gradually increased until, at a level of 6.0×10^3 U per kilogram per day, the polymerase level fell considerably. In Case 3 (Fig. 3), an intermediate dose of 1.2×10^4 U per kilogram per day also led to a prompt fall in polymerase activity. Comparison of the reduction in polymerase level produced in Case 3 to that observed in Case 1 (courses A and B) suggests that the higher dosages had a more complete effect. Case 4 (not included in the figures), who had no detectable polymerase before interferon therapy, remained polymerase negative throughout a one-month course of drug given at a dosage of 7.5×10^3 U per kilogram per day. Human leukocyte interferon was added to polymerase-containing serum in vitro at concentrations of 1000 and 500 units per milliliter. No effect on polymerase activity was observed when such a mixture was incubated at 37°C for two hours and room temperature overnight.

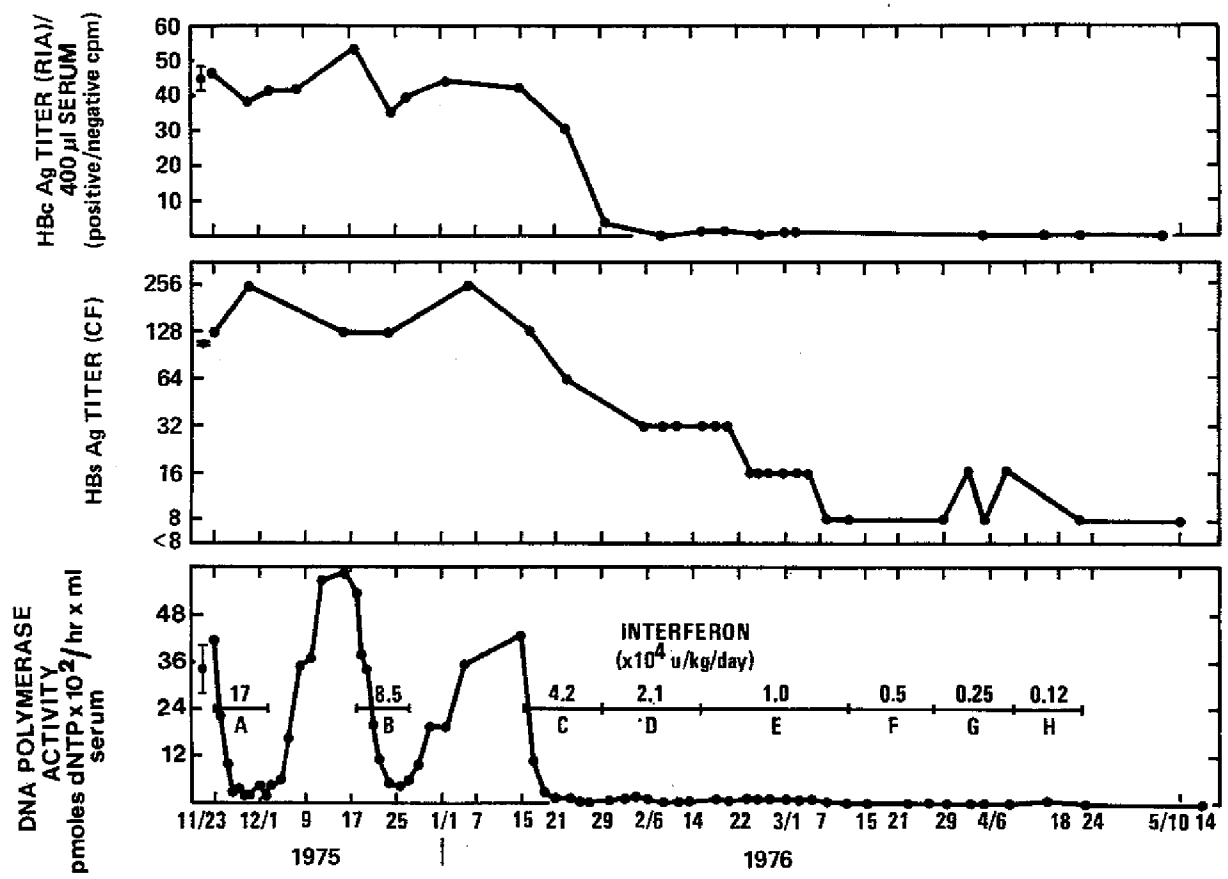

Figure 1. Effect of Three Separate Courses (A, B and C–H) of Human Leukocyte Interferon on Dane-Particle-Associated DNA Polymerase, HB_s Ag (by Complement Fixation) and HB_c Ag (by Radioimmunoassay) in Case 1.

ǂ denotes mean and 2 standard deviations of a minimum of four separate values in serum samples obtained at regular intervals during the two months before study. Letters A through H denote individual interferon treatment courses at specific dosages with the units of interferon per kilogram per day × 10^4 as shown.

Interferon, when given for 10 days or less at a dosage range between 17 × 10^4 (Case 1, Fig. 1), and 1.2 × 10^4 U per kilogram per day (Case 3, Fig. 3), was associated with only a transient fall in polymerase. When therapy was stopped, polymerase values promptly rose toward pretreatment levels. More prolonged therapy (Fig. 1 and 2) at dosages of ⩾6.0 × 10^3 U per kilogram per day appeared to suppress polymerase activity more permanently. Cases 1 and 2 continued to have depressed or negative polymerase values for nine weeks and 15 weeks respectively after the termination of therapy.

Other Dane-particle markers, including HB_c Ag and Dane-particle-associated DNA, appear to be similarly affected. In Case 1 (Fig. 1), HB_c Ag fell only slightly and transiently during courses A and B, but became undetectable during more prolonged treatment (courses C to H). Mixing of equal volumes of serum from course E (no detectable HB_c Ag) with precourse A serum (HB_c Ag ratio 41) did not alter HB_c Ag titer, implying that a serum inhibitor (anti-HB_c) was not responsible for the change in titer. In Case 2 (Fig. 2), a concurrent fall of HB_c Ag and polymerase was observed. Again, in these two subjects, after prolonged interferon therapy, HB_c Ag remained down. Case 3 (Fig. 3), who was treated for eight days, showed only a transient fall in his HB_c Ag. Case 4 had no detectable HB_c Ag throughout the study. Interferon affected Dane-particle-associated DNA in the same fashion as DNA polymerase activity (Cases 1, 2 and 3, Table 1).

The effect of interferon therapy on HB_s Ag, a viral protein not exclusively associated with Dane particles, appeared to be more variable. In no case did it appear that short-term interferon (Cases 1 and 3, Fig. 1 and 3) had a measureable effect on HB_s Ag levels. Of the three patients treated for a month or longer (Cases 1, 2 and 4, Fig. 1 and 2), only Cases 1 and 2 had noteworthy falls in HB_s Ag level. In Case 1, who received the highest doses of interferon, HB_s Ag fell 16-fold, from a complement-fixation titer of 1:128 to 1:8. Case 2 had a reproducible four-fold fall in HB_s Ag and Case 4 (not shown) had a constant HB_s Ag titer of 1:32 throughout the study.

e antigen, a hepatitis-B-associated, soluble antigen in the blood, was studied in the four patients. Short-term interferon therapy (Cases 1 and 3, Table 1) did not appear to affect this antigen. Prolonged interferon dosing (Cases 1 and 2, Table 1) was associated with a disappearance or a diminution of e antigen.

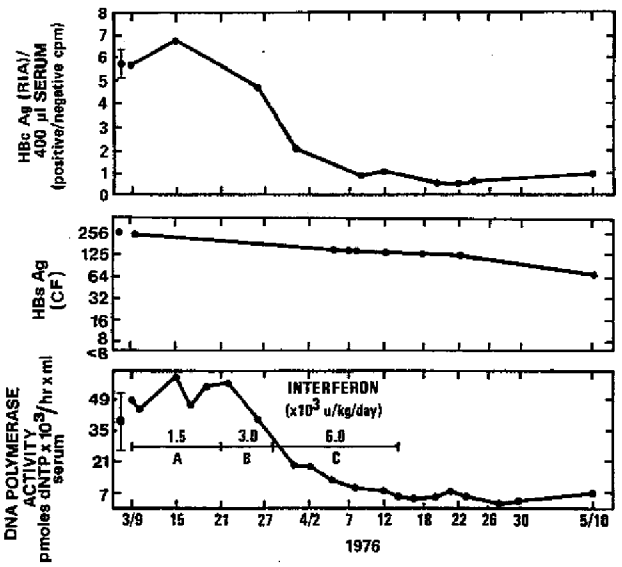

Figure 2. Effect of Three Separate Courses (A, B and C) of Human Leukocyte Interferon on Dane-Particle-Associated DNA Polymerase, HB_s Ag and HB_c Ag in Case 2.

Ŧ denotes mean and 2 standard deviations of a minimum of four separate values in serum samples obtained at regular intervals during the two months before study. Letters A through C denote individual interferon treatment courses at specific dosages with the units of interferon per kilogram per day × 10^3 as shown.

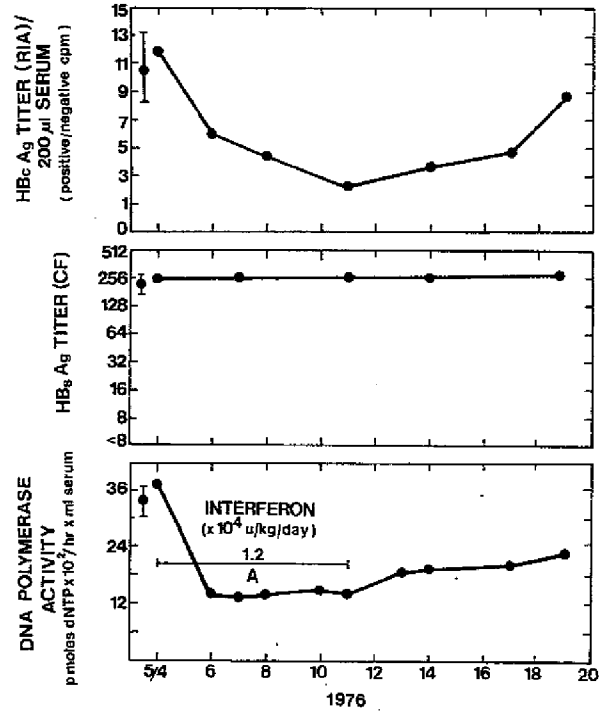

Figure 3. Effect of One Course (A) of Human Leukocyte Interferon on Dane-Particle-Associated DNA Polymerase, HB_s Ag and HB_c Ag in Case 3.

Ŧ denotes mean and 2 standard deviations of a minimum of four separate values in serum samples obtained at regular intervals during the two months before study. Letter A refers to a single interferon course.

Case 4 had no detectable antigen but was anti-e positive.

Immunologic Measurements

All patients were HB_s Ag positive throughout the study, and in no case was anti-HB_s detectable with use of a sensitive hemagglutination assay (Table 1). All chronic carriers have high levels of anti-HB_c.[24] This observation also held true in the four patients treated with interferon. Anti-HB_c titer did not appear to be consistently affected by therapy (Table 1). Anti-e was not detectable in Cases 1, 2 and 3 and did not appear to be affected by interferon in Case 4.

Table 1. Effect of Interferon on Various Virologic and Immunologic Indexes of Hepatitis B Virus (HBV) Infection.

Stage of Study	HBV Dane-Particle-Associated DNA (µg × 10^3/ml)	e Antigen	Anti HB_c	Anti HB_s	Anti e
Case 1:					
Before interferon course:	17.0	+	8×10³	<1:10	–
A	1.2	ND*	ND	ND	ND
(post-A)	14.0	ND	ND	ND	ND
B	2.1	±	ND	ND	–
C	0.3	–	ND	ND	–
D	<0.3	ND	ND	ND	ND
E	<0.3	–	16×10³	<1:10	–
Case 2:					
Before interferon course:	6.2	+	16×10³	<1:10	–
A	6.2	ND	ND	ND	–
B	0.45	±	ND	ND	–
After interferon	<0.3	–	64×10³	<1:10	–
Case 3:					
Before interferon course:	28.0	+	128×10³	<1:10	–
A	19.0	ND	ND	ND	ND
After interferon	18.0	+	128×10³	<1:10	–
Case 4:					
Before interferon	<0.3	–	16×10³	<1:10	+
After interferon	ND	–	8×10³	<1:10	+

*Not determined.

Liver Function and Toxicity

Interferon therapy was well tolerated in all patients. In Case 1 low-grade fever (temperature of 38.5°C) and mild malaise developed during courses A and B (17 and 8.5 × 10^4 U per kilogram per day). At the lower dosage levels, Case 1 as well as Cases 2, 3 and 4 had no observable symptoms. Whether interferon affects liver-function tests is not certain. Case 1, who had had relatively stable transaminases before study (Table 2), showed a rise and fall in both transaminases over a period of two weeks during course C. Case 2, whose liver-function tests had been highly variable before therapy, also had a transient elevation in transaminases during course C (Table 2). Cases 3 and 4 had no definite changes observed. Bilirubin and alkaline phosphatase remained within normal limits in three patients throughout the study. In Case 2, bili-

Table 2. Effect of Interferon on Hepatic and Hematologic Function, with Serum Interferon Levels.

Stage of Study	Serum Aspartate Amino-transaminase* U/ml	White-Cell Count	Peak Serum Interferon Level U/ml
Case 1:			
Before interferon course:	47 - 70 (50)†	8,100 - 9,400 (8, 500)‡	<10
A	41 - 69 (51)	3,400 - 4,000 (3,900)	1,250
B	41 - 47 (43)	3,600 - 4,800 (4,400)	—
C	130 - 294 (218)	5,000 - 5,500 (5,200)	210
D	119 - 165 (139)	4,900 - 5,600 (5,200)	89
E	75 - 159 (102)	5,300 - 7,700 (6,400)	50
F	52 - 74 (63)	6,000 - 7,100 (6,600)	23
G	35 - 52 (44)	7,200 - 8,100 (7,600)	16
H	31 - 35 (33)	8,100	—
Case 2:			
Before interferon course:	293 - 1790 (896)†	10,300 - 14,300 (12,700)‡	<10
A	117 - 239 (218)	10,300 - 13,300 (11,800)	10
B	251	9,800	23
C	463 - 576 (50)	8,600 - 10,400 (9,200)	50
Case 3:			
Before interferon course:	51 - 59 (55)†	4,300 - 5,800 (5,100)‡	<10
A	87 - 93 (90)	3,200 - 3,700 (3,500)	47
Case 4:			
Before interferon course:	25 - 43 (44)†	4,900 - 7,200 (6,100)‡	<10
A	33 - 46 (40)	4,100 - 5,100 (4,800)	45

*Normal value <15.

†Range, with mean of ≥4 determinations over previous 2 mo in parentheses.

‡Range, with mean of ≥2 determinations over previous 1-2 mo in parentheses.

rubin and alkaline phosphatase remained approximately 1.2 to 1.5 times normal throughout the study.

The effect of interferon on the hematologic system was more obvious. All four patients showed a transient depression in white-cell count (Table 2) as well as platelets and reticulocyte counts (not shown) during therapy. The effect was noticeable when peak serum interferon levels were in the range of 50 units per milliliter or higher. This finding corresponded to a dosage level of approximately 1×10^4 U per kilogram per day. The effect appears to be dose related (Table 2) and drug dependent in that all hematologic measurements promptly reverted to normal when interferon was stopped. Platelet counts fell to a low of 80,000 in Case 4 and not below 100,000 in the others. Hematocrits did not change during therapy.

Discussion

On five separate occasions in three patients, the parenteral administration of human leukocyte interferon was associated with a rapid fall in Dane-particle DNA polymerase activity. This effect was demonstrable over a dosage range between 6.0×10^3 and 17×10^4 U per kilogram per day and was seen in patients with both early and advanced liver disease. In individual patients, polymerase values tended to be constant over time. Thus, it seems highly unlikely that the reductions seen in this study occurred spontaneously. The falls in polymerase activity were accompanied by decreases in HB_c Ag as well as in Dane-particle-associated DNA. DNA polymerase, HB_c Ag and DNA are all constituents of the Dane particle, the putative complete hepatitis B viron.[17] The concentrations of these constituents as measured in this study are considered to reflect accurately the concentration of Dane particles in the serum. Interferon appears to be exerting a suppressive effect on the production of Dane particles. At what stage of Dane-particle production this effect occurs is not known; however, the effect on the Dane particles appears to be more regular and reproducible than that on the total HB_s Ag.

The effect of interferon on HB_s Ag production is not as striking. Short-term dosing did not affect surface-antigen titer in any of the patients. Treatment for one month or more was associated with a marked HB_s Ag fall in one of three patients and a lesser decrease in another. In Case 1, with the greatest response, interferon dosage was considerably higher, and treatment was continued longer than in the other patients (Cases 2, 3 and 4). Only a small fraction of the total circulating HB_s Ag is present in Dane particles; the great majority circulates freely as either 22-nm spheres or filamentous forms.[25] It is therefore not surprising that a profound change in Dane-particle concentration is not reflected in a change in HB_s Ag titer.

Immunofluorescent studies of infected hepatocytes have shown several patterns of hepatitis B antigen staining, including HB_s Ag in the cytoplasm and HB_c Ag in the nucleus, only cytoplasmic HB_s Ag and, less frequently, only nuclear HB_c Ag staining.[26,27] It is interesting to speculate that the interferon effect at the dosage ranges used in this study is greater on the cells producing HB_c Ag — i.e., the cells probably making complete virus. Cells making only excess surface antigen may be affected less.

In Cases 1 and 2, the suppressive effect of prolonged interferon therapy persisted well beyond the administration period. At present the duration and implications of this change are unknown.

Although the mechanism of the antiviral effect of interferon has been closely studied in a number of in vivo and in vitro systems,[28] there is at the moment no exact explanation for the effects produced in the hepatitis B virus infection. In addition to antiviral properties, interferon has recently been shown to modulate both humoral and cellular immunity.[29] Whether the effect of interferon on the hepatitis virus is directly antiviral or immune mediated or a combination of both remains to be elucidated.

In the present studies no definite effect on liver disease was observed. In the light of the variable progression of chronic active hepatitis, the small numbers of patients studied and the short duration of observations, the lack of definitive change in hepatic function is not surprising. Only after an optimal viral-suppres-

sive regimen is defined and a controlled double-blind study undertaken will it be possible to evaluate the therapeutic efficacy of interferon in the treatment of hepatitis-B-virus-associated liver disease. Mild reversible hematopoietic suppression was observed when peak serum levels reached approximately 50 U per milliliter. This effect has also been seen recently in patients with herpesvirus infections or neoplasia (or both) being treated with high-dose interferon (Merigan TC: unpublished data). It is not clear whether the suppression is an intrinsic property of interferon or caused by a contaminating protein. The suppression effect appears to be readily reversible when interferon is stopped and has not been a clinical problem at the dosage level active against the hepatitis B virus.

There is no direct evidence that the Dane particle is the infectious agent of hepatitis B. Indirect evidence supporting this conclusion includes the size and appearance of the particle,[13] the presence of a small double-stranded circular DNA in the core of the particle,[17] the demonstration that Dane-particle DNA will only hybridize with DNA from infected livers (Lutwick LI, Robinson WS: unpublished data) and the reaction of virus neutralizing antibody (anti-HB_s) with the surface of the Dane particle.[21] A recent report by Okada et al.[10] has shown a close association between Dane particles, circulating HB_c Ag, e antigen and chronic carrier mothers who transmit hepatitis B virus to neonates. The suppressive effect of interferon on a number of Dane-particle markers, including DNA polymerase, specific Dane-particle-associated DNA and circulating HB_c Ag, as well as its apparent effect on e antigen, suggests that interferon therapy might have lowered the infectivity in the blood of the treated patients. Chimpanzee transmission studies with serum obtained before and after interferon therapy could directly test this idea.

We are indebted to Ms. Janet Kahle and Barbara Hofmeister for technical assistance and to Drs. Michael Knauer and William Targgart for patient referrals.

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TAB C

GUEST EDITORIAL

Robert M. Friedman[1]

Interferons and Cancer[2]

Although interferons were discovered in 1957 (*1*), we do not yet fully understand their production, mechanism of action, biologic role, or therapeutic potential. Originally described as antiviral agents, interferons have other activities which include regulation of cell growth (*2*) and regulation of the immune response (*3*). Several recent findings have made interferons of current special interest to oncologists. Through the persistent effort of Dr. Kari Cantell, Central Public Health Laboratory, Helsinki, Finland, an amount of human leukocyte interferon sufficient for meaningful clinical studies of the effects of interferon on a few diseases has finally become available. Employing this interferon, a group at Stanford University, Stanford, California, under the direction of Dr. Thomas Merigan, has obtained evidence suggesting that human interferon may be useful in the treatment of chronic hepatitis B virus infection (*4*) and has found that interferon treatment of herpes zoster complicating leukemias and lymphomas has led to more rapid resolution of lesions and prevention of systemic effects (*5*). In addition, preliminary results from the laboratory of Dr. Hans Strander (Karolinska Institutet, Stockholm, Sweden), who has been treating 28 osteogenic sarcoma patients with Cantell's human interferon, suggest that the group receiving interferon has a lower rate of metastasis than does a concurrent control group (*6*). Strander's studies, although not yet involving sufficient numbers of patients to be highly significant, appear to warrant serious consideration of human interferon as an antitumor agent; animal studies supply strong evidence that in some situations interferons are effective in inhibiting tumor growth (*7*).

Interferons are proteins. Their production by animal cells can be induced by various substances (*1*), and they in turn inhibit a wide spectrum of RNA and DNA viruses by inducing an antiviral state. However, interferons are generally animal species-specific in their range of antiviral activity; only human interferons have been shown to be effective in treatment of human cells, although it is certainly possible that some animal interferons will prove active.

INTERFERON PRODUCTION

Interferon production is an induced activity. Under normal conditions animals produce interferons as one of a series of responses to virus infections, and interferons seem to be important in recovery from some primary virus infections (*7*). Lymphocyte interferons are lymphokines, inasmuch as their production may be induced by exposing macrophages and T-lymphocytes to mitogens or antigens (*8*). Interferons may also be induced in tissue cultures by double-stranded RNA forms such as polyriboinosinic-polyribocytidylic acid (poly I·poly C) and in animals by, for example, various intracellular parasites (bacteria, metazoa, protozoa), polymers, endotoxins, or double-stranded RNA forms (*9*).

When exposed to an interferon inducer, cells in culture make an interferon messenger RNA, which may be translated in cell-free systems or in frog oocytes (*10*, *11*). The amount of interferon produced by cells is proportional to the amount of interferon messenger RNA induced (*12*). The amount of messenger RNA may be increased by a superinduction procedure which involves treatment of cells with inhibitors of RNA and protein synthesis, after exposure to an inducer but before collection of interferon begins (*13*). The superinduction procedure appears to prolong the half-life of the interferon messenger RNA (*12*). The production of interferon presents the cell biologist with an interesting induction system in which negative feedback controls operate and an easily characterized protein is made.

ANTIVIRAL ACTIVITY OF INTERFERONS

Although the mechanism of action of interferon is not yet known, it seems to be the result of a complex series of events (*14*). Interferons bind on the cell surface to a specific receptor which seems to contain both ganglioside and peptide components [(*15*); Kohn LD:

ABBREVIATION USED: MuLV = murine leukemia virus.

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Editor's note: Periodically, the Journal publishes solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.

Unpublished data]. Binding of interferon to a cell surface receptor does not necessarily result in the development of antiviral activity, but in sensitive cells, i.e., those with the proper effector apparatus, binding causes chemical, morphologic, physical, and immunologic alterations in the plasma membranes (*16–18*). These alterations, possibly through a mechanism involving cyclic AMP (*19, 20*), seem to result in the production of an enzyme precursor which when activated can produce an intracellular antiviral substance (*21*). This enzyme is a protein, the production of which probably requires the synthesis of a new species of messenger RNA (*22*).

After an interferon-treated cell is infected with a virus, the production of a viral product, most likely a double-stranded form of viral RNA, seems to trigger the activation of the enzyme precursor (*23, 24*). The activation may involve phosphorylation of the precursor by a kinase also activated by a viral double-stranded RNA. The antiviral substance formed in cell-free extracts is a recently described adenosine oligomer with an unusual 2′,5′ linkage (Kerr IM, Ball LA: Personal communication). This antiviral substance may inhibit virus replication by several mechanisms, which possibly include inhibition of virus-directed transcription, or translation, or ribonucleolytic cleavage of viral messenger RNA.

SITE OF ANTITUMOR ACTIVITY OF INTERFERONS

Several hypotheses have been advanced to attempt to explain a putative antitumor activity of interferon. These include inhibition of tumor virus replication and cell transformation by virus and inhibition of tumor growth, directly or indirectly, through primary effects on the immune system.

Interferon was thought to inhibit tumor viruses through the same mechanism as that involved in the inhibition of other viruses (*14*). For the inhibition of both the DNA and RNA tumor viruses studied most completely, however, the mechanism of interferon action seems to have unusual features. For simian virus 40, the accumulation of early viral messenger RNA is inhibited (*25*), whereas in the best studied of other systems, early viral messenger RNA is not reduced by interferon treatment (*26*). Even more surprising were the findings on interferon inhibition of MuLV replication, a system in which interferon treatment does not seem to inhibit MuLV-directed protein or RNA synthesis (*27, 28*). Instead, defective virus with markedly reduced infectivity is synthesized (*29–31*). In some systems, the MuLV produced in interferon-treated cells is so defective that it cannot bud from the cell surface (*32, 33*). At least two abnormalities have been found in the defective MuLV released from interferon-treated cells: a large glycoprotein, possibly a precursor to the viral gp69/71 in the viral membrane (*34*), and a defective p30 reverse transcriptase complex with decreased enzyme activity (Bandyopadhyay AK: Personal communication).

Whatever the mechanism, however, interferon treatment results in a marked decrease in the production of infectious virus and in cell transformation by virus (*35*). Interferon may thus prevent virus recruitment of new cells into the transformed state, which may explain the repeated observations in many systems that interferon treatment inhibits induction of tumors in mice by oncogenic viruses (*2*). Interferon treatment also inhibits the growth of established virus-induced mouse neoplasms and has been successfully employed to inhibit transplantable tumors and chemically induced neoplasms in mice (*2*). Less success was usually attained in the treatment of solid transplantable tumors than in treatment of ascites tumors; however, interferon treatment inhibited the development of both the subcutaneous nodules of Lewis lung carcinoma at the site of transplantation and the development of pulmonary metastases from the transplant (*36*). Interferon administration reduced the incidence of fibrosarcomas and lung adenomas induced in CF_1 mice by 3-methylcholanthrene (*37*). These transplantable or induced tumors are not obviously due to viruses. Their inhibition by interferon suggested that interferon treatment might have a direct effect on tumor growth.

The inhibition of growth of tumors which apparently are not virus induced gave rise to speculation that interferon might be a growth control regulator and that the effects on tumors are caused by rapid tumor growth. If interferon does regulate the growth of cells, interferon treatment should show some effects on normal (nontumor) cells. In some studies, this has proved to be so, and in vitro interferon inhibited cell DNA synthesis and replication (*38*). Interferon treatment inhibited the regeneration of the liver in partial hepatectomized mice (*39*). Newborn C3H or Swiss mice treated with high concentrations of partially purified interferon exhibited weight loss and diffuse hepatic cell degeneration (*40*). Similarly, in a newborn child treated with human interferon for congenital cytomegalovirus infection, significant weight loss was recorded before the therapy was halted (*41*).

A third possible site for interferon-induced inhibition of tumor growth is the immune system. In both in vivo and in vitro studies, interferon treatment inhibited the antibody response (*42*). This effect of interferon was dose-dependent and could not be separated from the antiviral activity of the interferon preparations. Interferon also inhibited cellular immune responses and delayed-type hypersensitivity (*43*). Similar studies also showed an inhibition by interferon treatment of in vitro mitogen-stimulated DNA synthesis by T-lymphocytes (*44*). In mice, interferon treatment inhibited the response to allografts (*45*), sensitization to picryl chloride (*43*), and the delayed-type hypersensitivity response to sheep red blood cells (*46*).

Possibly related to studies on the immune system was the finding that interferon treatment inhibited phagocytosis (*47*) in mice and caused several specific alterations in the surfaces of lymphocytes. Interferon-treated lymphocytes had an increased capacity to absorb alloantisera (*17*) and an enhanced expression of surface H-1 antigens (*48*).

Thus interferon treatment might inhibit tumor growth in several ways through primary effects on the immune system. Alterations induced by interferon in the plasma membranes of lymphocytes might be responsible for an increased cytotoxicity. Interferon treatment could also alter the surfaces of tumor cells (as it does in lymphocytes and L1210 cells) to increase the expression of tumor-specific transplantation antigens. The increased phagocytosis induced by interferon treatment could have a role in the inhibition of tumor growth by causing rapid destruction and elimination of tumor cells. Finally, the inhibitory effects on antibody production could cause a decreased level of tumor-protective (blocking) antibodies, thus permitting the immune system to deal more effectively with tumors.

ATTEMPT AT A UNITARY HYPOTHESIS TO EXPLAIN THE VARIOUS ANTITUMOR EFFECTS OF INTERFERON

Many diverse effects of interferon treatment have been discussed so far. These include inhibition of viral RNA and protein synthesis, formation of defective virus particles, inhibition of normal and tumor cell growth, and inhibition of various aspects of the immune response. Can all of these activities be explained by interferon action at a single site? This is a difficult synthesis, but it is possible that changes induced by interferon on the plasma membranes of cells might be common initial sites of action. Antiviral action seems to be started by the binding of interferon to plasma membrane receptor sites and then, in sensitive cells, its affecting activation sites. These steps appear to cause several alterations in the plasma membrane. For RNA tumor viruses, the interferon-induced alterations in the plasma membrane could be responsible, at least in part, for the structural defects in virus produced by these cells. For other viruses, the early events (binding and activation) may be responsible for the intracellular activities that later lead to the inhibition of virus growth.

Alterations in the plasma membrane could also help to explain interferon's inhibitory action on normal and tumor cell growth. The phenomenon of contact inhibition of growth would suggest that events at the cell surface are important in determining the replication rate of cells. Interferon-induced changes in the plasma membrane could signal a cutback in the growth rate of malignant cells.

Finally, many of the effects of interferon on the immune system could be related directly to effects on the plasma membrane. Certainly, as discussed above, most of the effects on the immune system which may be related to the antitumor activity of interferons are membrane-associated events. These include increased phagocytosis, an increase in expression of transplantation antigens, and an increase in cytotoxicity of interferon-treated lymphocytes. Therefore, although it is a very speculative hypothesis, the actions of interferons may be due to a primary effect on the plasma membranes.

WHAT CAN BE DONE?

In spite of the promise of the investigations outlined above, the number of researchers in the field of interferon studies is small, and support for basic research has been modest. There are several reasons for this situation. Because of its great specific activity (one unit has been recently estimated to be less than 10^{-9} mg of protein), interferons have been difficult to obtain in large enough quantities for purification or for a significant number of clinical studies. In addition, assays for interferon are biologic, based on the ability of a preparation to inhibit virus replication; they are time-consuming and relatively inaccurate. Finally, in any one species several substances appear to have the general properties of interferons. For example, human white cell interferon preparations contain several molecular species of interferon (*49*). Interferon produced in human diploid cells has one component that antigenically resembles one of the interferons from white cells. Human T-lymphocytes, stimulated by mitogens or antigens, produce yet another distinct species of interferon. Although all of these interferons induce antiviral activity, it is not clear that they act in the same way.

It is no great wonder, then, that many editors and peer review groups are unenthusiastic about interferon research. Only because interferon had promise as an antiviral agent was it fortuitously included among the drugs to be studied by the Antiviral Substances Program of the National Institute of Allergy and Infectious Diseases, Bethesda, Maryland. For several years, under the excellent supervision of Dr. George Galasso, this program was probably the greatest source of funds for interferon research in this country. In many areas of interferon research, the Antiviral Substances Program was responsible for keeping up some momentum at a time when progress would otherwise have been stalled. At present, however, I believe that the exciting research leads on the molecular biology of interferon production and action, together with the preliminary data, discussed above, on the clinical uses of human interferon, should stimulate cancer researchers with a wide range of interests to consider doing studies in these areas. Also, large-scale bioengineering techniques should be used in the attempt to deal with the problems of interferon production and purification.

Such efforts may lead to solutions of basic problems in the understanding of biologic controls of animal cells. In addition, the leads that Gresser (*2*) and Strander (*6*) have put forth may be confirmed, and interferon could prove to be useful in the therapy of cancer.

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TAB D

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The production of interferon by 'genetic engineering'

Since the discovery of interferon in 1957[1], a number of attempts have been made to demonstrate the effectiveness of the product in the clinic[2]. Initially interest focussed around its use as an anti-viral compound but when it was realised that its antiviral action was not its only effect on cells interest turned to interferon as an anti-cancer agent. Material is now being used in clinical trials against both viral diseases[3] and cancer[4] and it is not inconceivable that it will also be used in the future as a modulator of the immune system[5] eg in tissue graft situations.

Interferon is a small polypeptide which is produced by cells in response to various inducers[6]. The activity of interferon is highly species specific[7] and hence only material produced from human cells (or monkey cells) in culture can be used for clinical studies. Human interferon is usually produced from either leukocytes[8] or from fibroblast cells[9]. Up to now, clinical studies have employed human leukocyte interferon. One cell is able to make only minute quantities of interferon so large quantities of human cells are needed for production. The problem of shortage of human interferon for clinical studies is still the major drawback. It is for this reason that some people have turned their attention to a line of transformed human lymphblastoid cells[10]. These cells can be grown in very large numbers and hence larger quantities of interferon can be produced. However, probably the best way to produce large quantities of interferon will be to introduce the gene(s) for interferon production into bacteria[11]. This technique has already been demonstrated with other mammalian genes eg somatostatin[12], insulin[13], ovalbumin[14], dihydrofolate reductase[15] and hepatitis antigen[16].

Since almost all eukaryotic genes examined to date contain interruptions (introns) within their coding sequences[17], one problem

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is to isolate the gene sequence free of these introns. This has been done either by making a reverse transcript of the messenger RNA[18] or by making a chemically synthesised copy of the gene[19]. It is possible to consider both these options for human interferon.

The existence of interferon mRNA was first demonstrated by De Maeyer-Guignard *et al*[20] who used a heterologous cell system for mRNA translation. Since then interferon mRNA has been translated in a variety of cell-free translation systems[21] and in oocytes from *Xenopuslaevis*[22]. Purification methods for mRNA are well documented and include such techniques as oligo(dT)-cellulose (or polyU-sepharose) chromatography[23], fractionation by sucrose gradient sedimentation[24] and immunoprecipitation of polysomes[25]. Considering this latter possibility, one pre-requisite for this is to have available good high titre mono-specific antibody. Since human interferon has only been purified to homogeneity in very small amounts, this antibody cannot be raised by normal injection procedures. However the recently reported techniques for raising monoclonal antibodies involving the fusion of myeloma cells with antibody producing cells[26] should provide a system for producing high titre mono-specific antibody to human interferon.

In addition to the above standard purification techniques for mRNA, the purification of interferon mRNA has one extra possibility. Interferon mRNA is inducible[27] and hence if mRNA from such induced cells is hybridised to cDNA made from the mRNA from non-induced cells, then there will be a significant enrichment for interferon specific sequences.

Once interferon mRNA is available in reasonable quantities in a pure or partially pure form, the techniques for producing a double stranded cDNA[28] are well documented. This ds cDNA can then be inserted into a suitable vector plasmid using a variety of available methods including biochemical tailing of the molecules with terminal transferase[29], the use of synthetic linker[30] molecules or directly by blunt end ligation[31]. One might design the system such as to optimise the chance of expression of the sequence on the vector of choice eg by inserting the cDNA into the PstI site of pBR322 by dG-dC tailing[32] or using any other systems in which expression might be demonstrated[33]. In order to demonstrate expression of interferon by the bacterial cultures antibody techniques can again be used[34]. The results of this can then be confirmed by looking at the biological activity of the protein produced[35].

Although initially *E coli* and its plasmids would be the natural choice of host/vector system for this work, there is no reason why other microorganism host/vector systems should not be used[36]. These could include other bacteria, fungi or another possibility would be the use of a yeast[37] system which being eukaryotic may lead to easier expression of the sequences. It is also conceivable to use an animal cell/virus, host/vector system. Although this does not appear at first glance to offer significant advantages over the normal cell culture production methods, it would probably lead to higher yielding systems because of the increased gene copy number on a virus vector. Animal viruses have been successfully used as cloning vehicles and demonstrated to express inserted β globin sequences[38].

Even if expression could not be demonstrated, the presence of interferon cDNA in the hydrids can be demonstrated in a number of ways. For example, the differential hybridisation (by the Grunstein-Hogness method[39] or similar protocols) with radiolabelled probes from induced and non-induced cells will identify clones which may contain interferon cDNA sequences. If these are then isolated they can be hybridised to traditionally active interferon mRNA and screened for inhibition of this activity.. Only those clones which contain interferon cDNA will inhibit translation. Once such sequences are isolated, even if they only contain part of the interferon sequence, they can be used in a number of ways, eg to hybridise to interferon mRNA and be used a primers for extending the synthesis of the cDNA.

Small quantities of human interferon have been purified[40]. As yet although not enough pure material is available to determine the amino acid sequence of the protein, it is obvious that this will soon be achieved. It then becomes possible to chemically synthesise the gene[41] for interferon and to achieve expression of this in a bacterium in a similar way to that demonstrated for somatostatin[42].

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TAB E

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Maximizing Gene Expression on a Plasmid Using Recombination in Vitro

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Summary

Recombination in vitro has been used to place one or more copies of a strong promoter, the *lac* promoter, at varying distances from the *c*I (repressor) gene of bacteriophage λ on the E. coli plasmid pMB9. In all constructions, λ repressor synthesis is driven wholly or predominantly by the inserted *lac* promoter. One of our fusions directs the synthesis of very high levels of λ repressor. In this case, the fused DNA encodes a ribosome binding site which is a "hybrid" of λ and *lac* sequences. In principle, this method of construction should elicit high levels of expression in E. coli of any gene, whatever its source. We also described strains with different sequence arrangements that, for reasons not completely understood, produce less repressor.

Introduction

Synthesis of a protein at high rates in E. coli depends upon frequent transcription of the protein-encoding gene and efficient translation of the mRNA. Frequent transcription requires a "strong" promoter; efficient translation apparently requires that the mRNA bear a ribosome binding site. According to current models, a ribosome binding site on an E. coli mRNA includes the translational start codon AUG (or GUG) and another sequence that is complementary to bases on the 3′ end of 16S ribosomal RNA. Shine and Dalgarno (1975) first postulated the requirement for this homology, and various SD sequences, as we call them, have been found in almost all E. coli mRNAs examined. Identified SD sequences vary in length from 3-9 bases and precede the translational start codon by 3-12 bases (for review, see Steitz, 1977). One case is known in which an AUG translational start is located at the 5′ end of a mRNA, and that message is translated in vivo about an order of magnitude less efficiently than is another mRNA bearing a complete ribosome binding site (Ptashne et al., 1976; Walz, Pirrotta and Ineichen 1976).

Experiments reported in this paper utilize recombination in vitro to place one or more copies of a strong promoter, the *lac* promoter, at varying distances from the *c*I (repressor) gene of bacteriophage λ on the E. coli plasmid pMB9 (Rodriguez et al., 1976). In all constructions, λ repressor synthesis is driven wholly or predominantly by the inserted *lac* promoter(s). In our most potent fusion, we created a "hybrid" ribosome binding site; the *lac* promoter and adjacent sequences were positioned so that the *lac Z* gene SD sequence was 8 base pairs upstream from the ATG of the *c*I gene. In principle, this strategy should elicit high levels of expression in E. coli of any gene, whatever its source. We begin with a description of the construction and properties of the "hybrid" ribosome binding site strain. We then describe strains with different sequence arrangements that, for reasons not completely understood, produce less repressor.

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Results and Discussion

Expression of cI with a Hybrid Ribosome Binding Site Driven by the *lac* Promoter

Plasmid pKB280 is a derivative of pMB9 that carries a single λ *c*I gene fused to a single *lac* promoter. The *lac* DNA fragment used in this construction bore, in addition to the promoter, the *lac Z* SD sequence $\frac{\text{AGGA}}{\text{TCCT}}$ plus five additional bases. The remainder of the sequence coding for the *lac Z* gene, including the initiator ATG, was removed by cleavage with a restriction endonuclease. The fragment of λ DNA used included the sequence

```
        cI
5'  CGTATG . . .
3' TGCATAC . . .
```

plus adjacent sequences from *c*I. The *c*I SD sequence as well as all other sequences upstream from those described were removed by cleavage with a different restriction endonuclease (see Figure 1). Analysis of the DNA sequence of a portion of pKB280 (T. M. Roberts, personal communication) reveals that the *lac* promoter has been fused to *c*I so that the *lac Z* SD sequence is positioned eight bases from the *c*I ATG as follows:

```
                    cI
ACACAGGAAACAGCGTATG.
TGTGTCCTTTCTCGCATAC
```

Apparently the terminal 3′ T on the λ fragment was clipped off at some stage in the construction and the flush-ended *lac* fragment joined to the λ fragment excluding this base. In the case of *lac Z*, the SD sequence $\frac{\text{AGGA}}{\text{TCCT}}$ is separated from its ATG by 7 base pairs. In λ, the longer SD sequence $\frac{\text{GGTGAT}}{\text{CCACTA}}$ is separated from the *c*I ATG by 11 base pairs (see

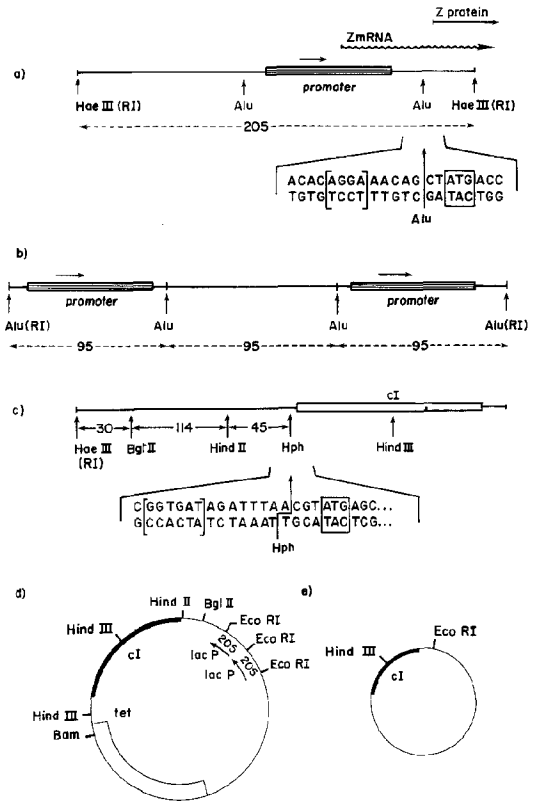

Figure 1. DNA Molecules Used in Constructions

In each case, pertinent restriction endonuclease cleavage sites are indicated. In some cases, Alu I and Hae III ends were converted to Eco RI ends by the method described in Backman et al. (1976), and these sites are designated Hae III (RI) or Alu I (RI). The distance between certain cleavage sites is given in base pairs.

(a) A (nominally) 205 base pair fragment bearing a *lac* promoter plus sequences extending through the first eight codons of the *lac Z* gene (Gilbert et al., 1975; Backman et al., 1976). The *lac* promoter bears the UV-5 mutation which renders it CAP-independent. The sequence near the beginning of *lac Z* shows that Alu I cleavage separates the SD sequences (in brackets) from the translational start codon ATG (boxed).

(b) A 285 base pair fragment that contains two 95 base pair *lac* promoter fragments separated by a 94 base pair heterolcgous fragment. The ends of the promoter-containing segments correspond to the Alu I sites in (a), and so each contains a *lac* SD sequence but no *lac Z* initiator codon. The arrows indicate that the promoters initiate transcription in the same direction. This promoter-bearing fragment was constructed by L. Johnsrud (personal communication) who made it available to us on plasmid pLJ3.

(c) A portion of the λ genome that includes the *cI* gene. The sequence shows that the restriction endonuclease Hph I cleaves between the *cI* SD sequences (in brackets) and the translational start codon (boxed).

(d) Plasmid pKB252 (Backman et al., 1976). Plasmid pKB252 is a derivative of pMB9 that contains a single λ *cI* gene and two *lac* promoters carried on the *lac* DNA fragment described in (a). The plasmid also carries the gene coding for resistance to tetracycline, as indicated. Plasmid pKB255, not shown here, is identical to pKB252 except that it carries only one *lac* promoter.

(e) Plasmid pKB166. This smaller plasmid carries the *cI* gene but not the *tet* gene nor any *lac* promoter (Backman et al., 1977).

Figure 1). [In the life cycle of phage λ, the *cI* gene is transcribed in two different modes. In a λ lysogen, the *cI* promoter is P_{RM}, and the mRNA begins at the 5′ end with the AUG that codes for the amino terminus of repressor. Upon infection of a nonlysogen, transcription of *cI* apparently initiates about 300 base pairs upstream from the gene at the promoter P_{RE}. The DNA sequence indicates that this mRNA contains a 6 base SD sequence (see Figure 1) that could direct efficient translation (for review, see Ptashne et al., 1976).]

An E. coli strain carrying pKB280 produces roughly 1% of its soluble protein as λ repressor. [The values given throughout this paper for the levels of λ repressor in strains bearing our various recombinant plasmids refer to the growth conditions listed in Experimental Procedures. These numbers may change if the growth conditions are changed. For example, J. Roberts (personal communication) reported to us, and we have confirmed, that cells containing the plasmid pKB252 grown in a low sulfur glucose-salts medium containing 20 μg/ml tetracycline produce some 10 fold higher repressor levels. Bailone, Levine and Devoret (1977) have made a similar observation using a different medium. We have not yet tested strain 280 under these conditions. Our most convenient repressor source is a strain bearing pKB277 (see Constructions in Experimental Procedures) which produces about 2.5% of its protein as repressor when grown in tryptone broth.] This corresponds to roughly 30,000 monomers of repressor per cell. We can approximate the efficiency of repressor production by pKB280 as follows: we assume that a single induced *lac Z* gene ordinarily maintains about 5000 monomers of β-galactosidase per bacterium (I. Zabin, personal communication). A plasmid closely related to pKB280 (pKB252, described below) is maintained in about 50–80 copies per E. coli chromosome in stationary cells (Backman, 1977). These numbers suggest that our strain bearing pKB280 produces about an order of magnitude less repressor than theoretically possible. We show below that the *lac* promoter functions with full efficiency when carried on a plasmid.

The *lac* Promoter Functions Normally on a Plasmid

Plasmid pKB252 (Backman, Ptashne and Gilbert, 1976) bears one λ *cI* gene and two *lac* promoters (Figure 1d). The promoter-bearing *lac* DNA fragments are inserted 187 base pairs from the *cI* ATG, and each includes the *lac Z* SD sequence, the *lac Z* initiation codon and sequences encoding the next seven *lac Z* amino acids. Thus translation begins at sequences near each *lac* promoter, and because there are no translational stops in the appropriate frame (see Figure 4 and Gilbert et al.,

1975), translation of messenger from these promoters continues into *cI*. Translation initiated at *lac* is not in phase with the initiator ATG of *cI*, and it presumably terminates in *cI*. Functional repressor is therefore synthesized by translation that initiates at the *cI* ATG. Apparently both *lac* promoters are functional, because plasmid pKB255, which is identical to pKB252 except that it bears only one *lac* promoter, directs the synthesis of about half as much repressor as does pKB252 (see Table 1). We have constructed a deletion derivative of pKB252 in which the distance between the beginning of *cI* and the *lac* promoters was shortened by 114 base pairs. This plasmid, pKB265, directed the synthesis of repressor levels indistinguishable from those of its parent.

Bacteria bearing either pKB252 or pKB265 produce about 3–5 fold less repressor than do strains bearing pKB280. We considered two explanations for the relatively low yield of repressor from strains bearing pKB252 or pKB265: transcription from the *lac* promoter is impaired on the plasmid, and/or translation of *cI* is inefficient presumably because of interference caused by translation initiated upstream at sequences near the *lac* promoter. The properties of strains bearing a recombinant phage described in the following paragraphs reveal that the *lac* promoter functions normally in pKB252 and pKB265, but that the *cI* gene is translated inefficiently.

Phage λKB1 contains a single *lac* promoter fused to a λ *cI* gene (see Figure 3). In this phage, transcription initiating at the *lac* promoter traverses *cI*, part of *trp B*, all of *trp A* and then *lac Z*. The *lac Z* and *trp A* genes contain sequences coding for their wild-type ribosome binding sites, but transcription of these genes is strictly dependent upon the *lac* promoter located upstream of *cI*. Because the efficiency of translation of *lac Z* in the original *trp-lac* fusion used to construct λKB1 is similar to that observed for a wild-type *lac* operon (Reznikoff et al., 1974), the β-galactosidase levels in lysogens of λKB1 are a measure of transcription of *lac Z* initiated at the *lac* promoter in λKB1. In λKB1, the λ *cI*, *lac* and *trp* genes are substituted for nonessential phage genes, and the necessary heteroimmunity is provided by DNA from the related phage λ*imm*[21].

The levels of λ repressor and β-galactosidase in a strain lysogenic for λKB1 are given in Table 1. In the absence of IPTG, transcription from the *lac* promoter is repressed by *lac* repressor, and the levels of both repressor and β-galactosidase are low. When induced with IPTG, λKB1 directs the synthesis of about 1000 *lac Z* monomers per cell, but only about 80 λ *cI* monomers per cell. We draw three conclusions from these results. First, as expected, the *lac* promoter in λKB1 is apparently working at a level comparable to that observed in a wild-type *lac* operon. The difference between the value of 1000 monomers per cell seen with λKB1 and that of roughly 5000 monomers per cell observed in a typical *lac*$^+$ strain may be a result of attenuation of transcription between the *lac* promoter and the *lac Z* gene, which are separated by some 3000 base pairs in λKB1. Second, the *lac* promoter functions about as well on the plasmids pKB252 and pKB265 as in λKB1. This follows from the fact that 80 copies of pKB252 (or pKB265), each of which contains two *lac* promoters, direct the synthesis of somewhat more than 100 times as much repressor as does one such promoter contained on a single copy on λKB1. Third, the *cI* gene in λKB1, and apparently that on pKB252 and pKB265, is inefficiently translated, at best about 8% as well as the *lac Z* mRNA transcribed from the same promoter.

Table 1. λ Repressor and β-Galactosidase Levels in Various Strains

Strain	λ Repressor	β-Galactosidase
294 (λ) + IPTG	200	5000
W4680A (λKB1)	10	110
W4680A (λKB1) + IPTG	80	1000
294/pKB255	4,500	
294/pKB252	10,000	
RV suA/pKB252	9,800	
294/pKB265	7,500	
294/pKB268	2,900	
RV suA/pKB268	10,000	
294/pKB280	30,000	

Cells were grown in M9 glucose medium (or M9 glycerol medium where β-galactosidase values were reported) and assayed for λ repressor and in some cases β-galactosidase. λ repressor and β-galactosidase levels are reported in monomers of polypeptide per cell. The single lysogen level of λ repressor and the fully induced level of β-galactosidase are included for comparison purposes.

Why is *cI* translated so poorly in λKB1 and in the related plasmid strains? Three possibilities are that translation initiated near the *lac* promoter, which continues into *cI*, interferes with translation initiated at *cI*; that the *cI* SD sequence located adjacent to the *cI* ATG does not mediate efficient translation; and that *cI* mRNA containing the SD sequence must be processed to be translated efficiently, and this processing is abnormal in our fusion strains. There is no firm evidence that *cI* mRNA is processed, and we have no further comment on the third possibility. We cannot exclude the second possibility, but we doubt it for the following reasons: Gronenborn (1976) has constructed a *lac-cI* fusion in vivo in which the *lac* promoter is located

about a thousand bases from *c*I. In this case, translation of *c*I depends upon the *c*I SD sequence, and it is reported that in the presence of a transcriptional polarity suppressor (*su*A), a strain bearing this fusion produces some 40 fold more repressor than does a lysogen of λKB1. These findings indicate that the *c*I SD sequence promotes efficient translation of *c*I when carried on the appropriate mRNA. We designed a test of the first possibility, that in λKB1 and the related plasmid fusions, translation initiating near the *lac* promoter was interfering with translation of *c*I. This entailed constructing a fusion (pKB268) similar to pKB252, but differing in that the *lac* promoter-bearing DNA fragment lacked the *lac Z* initiating codon and structural gene codons.

Transcriptional Polarity Exposed by Eliminating Translation

Plasmid pKB268 bears one λ *c*I gene transcribed from two *lac* promoters. In this case, the ATGs which signal initiation of *lac Z* translation have been removed (see Figure 2). The two *lac* promoters are, however, inserted at the same position relative to *c*I as are those in pKB252. Table 1 shows that our standard E. coli strain bearing this plasmid produces some 3 fold less repressor than does the strain carrying pKB252 or pKB265. Apparently elimination of the translational initiation signal near the *lac* promoter has revealed a site (or sites) of transcriptional attenuation between the *lac* promoter and *c*I. This follows from the fact that when carried in a strain bearing a polarity suppressor (*su*A), pKB268 causes production of as much repressor as do pKB252 or pKB265. Our current understanding of transcriptional polarity suggests that polarity is observed only in the absence of translation (Adhya et al., 1976), and as expected, the polarity suppressor has no effect on repressor production coded by pKB252.

We cannot say exactly how efficiently the polarity suppressor we used (*su*A 115-1) functions in suppressing polarity in pKB268. This same suppressor has been found to suppress polar insertions in the *lac* operon with an efficiency of between 1 and 30%, depending upon the nature and location of the insertion (M. Malamy, personal communication). These numbers suggest that were polarity completely overcome in pKB268, repressor production would increase 3–100 fold above that seen in our *su*A strain. Moreover, as mentioned previously, pKB252, which is unaffected by the polarity suppressor, produces only about as much repressor as does the suppressed pKB268. These considerations suggest that in pKB252, translation initiated near the *lac* promoter decreases *c*I translation some 3–100 fold.

We note that the base sequence between the *lac* promoter and *c*I in pKB268 does suggest a possible source of transcriptional polarity. As shown in Figure 4, the mRNA transcribed from the *lac* promoter contains a region 64 bases long that can form a hairpin in which 50 bases are paired, leaving 5 unpaired bases as a loop. [The RNA forming this stem and loop is a transcript of the right operator (O_R) of λ.] Stem and loop structures have been implicated in transcriptional polarity in other cases (M. Rosenberg, D. Court, D. Wulff, H. Shimatake and C. Brady, manuscript submitted).

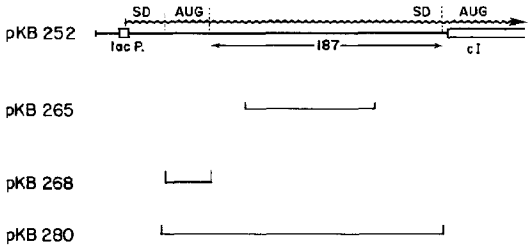

Figure 2. A Schematic Representation of Portions of Various *c*I Gene-*lac* Promoter Fusions

Three of the plasmids may be regarded as deletion derivatives of the fourth, pKB252. The brackets indicate the extent of each deletion. Note that although not indicated in this figure, pKB280 has one *lac* promoter, whereas the other plasmids have two.

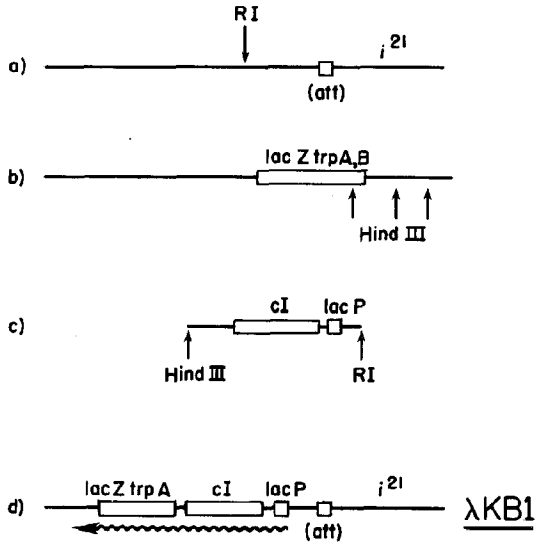

Figure 3. The Components of Phage λKB1

(a) Phage λRP167 (see reference 40 of Ptashne et al., 1976) which contains a single Eco RI cleavage site.
(b) A λ phage bearing the *lac-trp* fusion W205 (Barnes, Siegel and Renzikoff, 1974; Mitchell, Renzikoff and Beckwith, 1975). Cleavage with the restriction endonuclease Hind III generates a fragment bearing the left half of the phage chromosome plus the *lac Z, trp A,* and part of the *trp B* genes.
(c) A DNA fragment generated by partial digestion of pKB265 that carries the λ *c*I gene and one *lac* promoter.
(d) The assembled phage λ KB1. The arrow indicates the direction of transcription from the *lac* promoter.

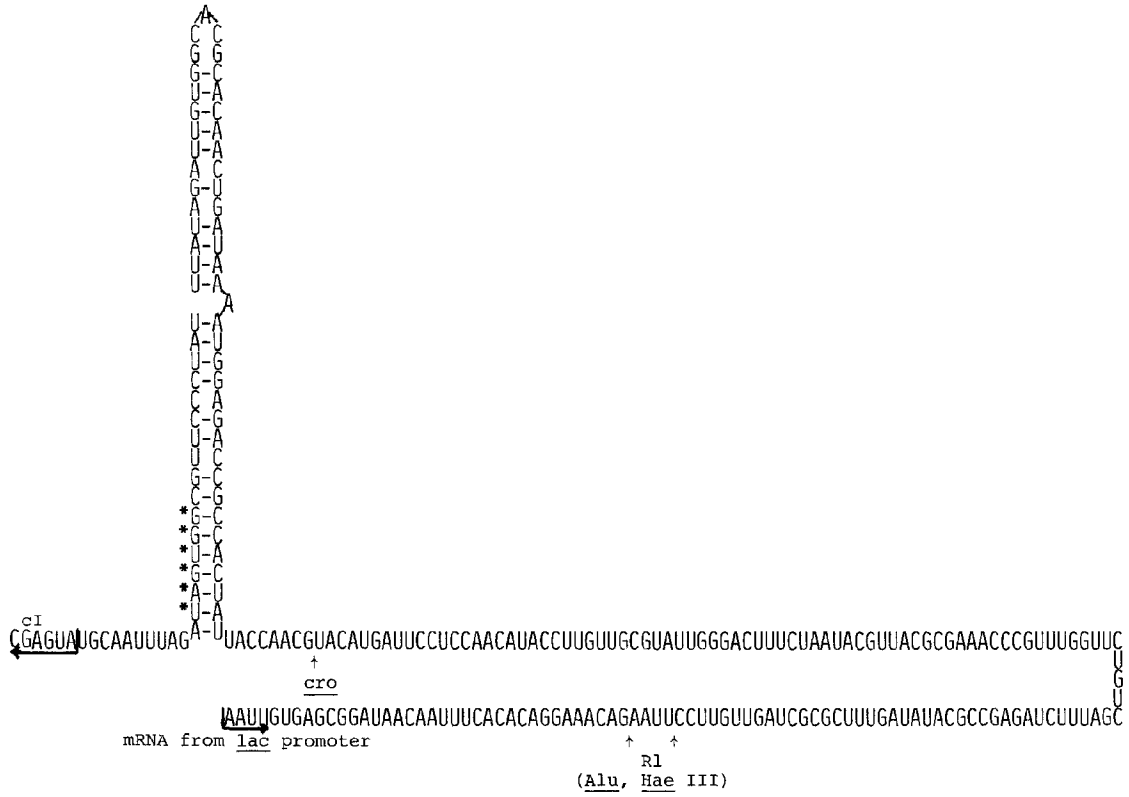

Figure 4. The Sequence of the RNA between the *lac* Promoter and *cI* in pKB268 (T. M. Roberts, H. Shimatake, C. Brady and M. Rosenberg, Manuscript Submitted; T. M. Roberts, Personal Communication)

The important feature is the large internal homology in the region just preceding *cI*. Note that the *cI* SD sequence is contained in this hypothetical stem and loop. Also indicated are various restriction endonuclease cleavage sites in positions corresponding to their sites in the DNA. The sequences between the arrow marked *cro* and the arrows indicating the Eco RI site are read from the antisense strand of the λ *cro* gene.

Conclusions

We have joined a sequence coding for the *lac* promoter and a portion of the ribosome binding site of the *lac Z* gene to a sequence containing the λ *cI* gene and the ATG that codes for the translational start of *cI*. We thus created a hybrid ribosome binding site bearing the *lac Z* "Shine-Dalgarno" sequence separated by 8 bases from the AUG of the *cI* on the mRNA. When carried on the plasmid pMB9, this fusion results in high level production of the λ repressor. It is possible that even higher levels of repressor production might be achieved by varying the distance between the two elements of the ribosome binding site. Our current understanding of the mechanism of ribosome binding of messages suggests that the only strong requirement for translation of a message in E. coli cells is that they contain a "Shine-Dalgarno" sequence near an initiating AUG or GUG, and that this ribosome binding site be accessible to ribosomes. [Weissman et al. (1977) find that mutation of the first base of the second codon of the Qβ coat protein cistron modifies ribosome binding (for further discussion, see Steitz, 1977).] If this is correct, our method should be applicable to eliciting efficient expression of genes from any organism in E. coli. If restriction endonuclease cuts are not suitably located just outside an initiator ATG, it should be possible to make the proper length molecules starting with longer fragments and trimming with exonuclease and single-strand-specific nucleases, or by synthesis in vitro of DNA molecules containing an initiator ATG and adjacent sequences which may be placed between an appropriate SD sequence and the remainder of the gene. Our experiments show that placing a strong promoter (the *lac* promoter) with or without a contiguous translational start signal, "near" a gene (the *cI* gene) does not necessarily result in efficient production of the gene product. Although transcription initiated frequently in our various fusions, in most cases one or another obstacle prevented maximal production of the gene product. The con-

struction of a hybrid ribosome binding site constituted a rational solution to the problem.

The failure of most of our fusions to produce high levels of repressor may indicate mechanisms that ensure high level repressor synthesis upon infection by phage λ. Transcription of *cI* initiated at P_{RE} (see Results and Discussion) proceeds through *cI* when supplied with the positive regulatory protein products of the phage genes *cII* and *cIII* (for review, see Herskowitz, 1973). It is not known whether translation is initiated near the transcriptional start on this long mRNA leader. If so, it presumably does not interfere with *cI* translation as does upstream translation initiated in our fusions of pKB252 and pKB265; if not, then the transcriptional attenuation revealed in plasmid pKB268 may be overcome as a result of *cII* and *cIII* action.

Experimental Procedures

Procedures for the construction and cloning of plasmids, the use of T4 polynucleotide ligase, DNA polymerase I, selection of clones, and assays of λ repressor and β-galactosidase were as reported in Backman et al. (1976). E. coli strain 294 (*endo* I, r_k^-, m_k^+, B_1^-; Backman et al., 1976), RV *su*A 115-1 (△ *lac* X74, B_1^-, *su*A 115-1; obtained from D. Ratner) and W4680A (△ *lac Z* 4680 B_1^-, *met*$^-$, *rec*A$^-$) were used as hosts. Cells were grown in M9 salts (Miller, 1972) plus 0.3% casamino acids, 0.01% thiamine hydrochloride and 0.5% glucose or glycerol as carbon source. Alu I, Bam I, Bgl II and Hph I restriction endonuclease digestions were performed in 6.6 mM Tris-HCl (pH 7.4), 6.6 mM $MgCl_2$, 6.6 mM 2-mercaptoethanol. Eco RI, Hae III, Hind III restriction endonuclease digestions were performed in the same buffer plus 50 mM NaCl. Strain W4680A was the host lysogenized with λKB1. Strain 294 was the host carrying the various plasmids unless stated otherwise.

Constructions

pKB280

An Eco RI-Alu I fragment (a), 95 base pairs long and carrying a *lac* promoter, was generated from the fragment shown in Figure 1b. A Hph I-Hind III fragment (b), 477 bases long and bearing most of *cI*, was isolated from pKB252. Plasmid pKB166 (Backman, Hawley and Ross, 1977) was cleaved with Hind III and Eco RI, and the larger fragment was isolated (c). Fragments (a), (b) and (c) were then ligated together in a single reaction. The Hind III end of (c) joined the Hind III end of (b); the Eco RI end of (c) joined the Eco RI end of (a); and the flush Alu I end of (a) joined the 1 base staggered Hph I end of (b). λ immune, *lac*-operator-containing clones were selected following transformation. A clone containing one plasmid, pKB277, directed the synthesis of about 2.5 fold more repressor than did pKB280 in our standard strains. We do not know the reason for this higher level of repressor synthesis. To compare this fusion fairly with our other plasmids, the *lac*-λ fusion was then recloned in pMB9 by excising the fusion by cleavage with Eco RI and Hind III, and cloning that fragment together with a Hind III fragment containing the carboxy terminus of *cI* between the Eco RI and Hind III sites on pMB9. This yielded pKB280.

pKB265

Plasmid pKB252 was cleaved with Bgl II and treated with DNA polymerase I to fill in the sticky ends. The plasmid was then cleaved with Bam I, and the two products were separated. The *cI*-bearing fragment was partially digested with Hind II, and the product resulting from cleavage only at the Hind II site shown in Figure 1d was isolated. This was then joined to the filled-in Bgl II-Bam I fragment containing the remainder of the plasmid. The desired product was formed by sticky-end joining of the Bam I ends and by flush-end joining of the Hind II and filled-in Bgl II ends. λ immune clones were isolated following transformation. Correct joining of the Hind II and filled-in Bgl II ends in pKB265 has been confirmed by DNA sequence analysis (B. Meyer, personal communication).

pKB268

The *lac*-promoter-containing fragment shown in Figure 1b was cloned in the Eco RI site of plasmid pKB158. The latter is identical to pKB252, except that it is missing the *lac* promoters, and its construction was described previously (Backman et al., 1976).

λKB1

The right half of the genome of phage λRP167 and the left half of the genome of phage λW205 were isolated following cleavage with Eco RI and Hind III, respectively (see Figures 3a and 3b). Partial digestion of pKB265 with Eco RI and Hind III yielded a fragment roughly 1350 bases long which includes the *cI* gene and a single *lac* promoter (see Figure 3c). The three pieces were joined by "sticky-end" ligation, and the composite phage was isolated by transfection.

Acknowledgments

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TAB F

Useful Proteins from Recombinant Bacteria

Bacteria into which nonbacterial genes have been introduced are able to manufacture nonbacterial proteins. Among the proteins made by recombinant-DNA methods are insulin and interferon

by Walter Gilbert and Lydia Villa-Komaroff

A living cell is a protein factory. It synthesizes the enzymes and other proteins that maintain its own integrity and physiological processes, and (in multicelled organisms) it often synthesizes and secretes other proteins that perform some specialized function contributing to the life of the organism as a whole. Different kinds of cells make different proteins, following instructions encoded in the DNA of their genes. Recent advances in molecular biology make it possible to alter those instructions in bacterial cells, thereby designing bacteria that can synthesize nonbacterial proteins. The bacteria are "recombinants." They contain, along with their own genes, part or all of a gene from a human cell or other animal cell. If the inserted gene is one for a protein with an important biomedical application, a culture of the recombinant bacteria, which can be grown easily and at low cost, will serve as an efficient factory for producing that protein.

Many laboratories in universities and in an emerging "applied genetics" industry are working to design bacteria able to synthesize such nonbacterial proteins. A growing tool kit of "genetic engineering" techniques makes it possible to isolate one of the million-odd genes of an animal cell, to fuse that gene with part of a bacterial gene and to insert the combination into bacteria. As those bacteria multiply they make millions of copies of their own genes and of the animal gene inserted among them. If the animal gene is fused to a bacterial gene in such a way that a bacterium can treat the gene as one of its own, the bacteria will produce the protein specified by the animal gene. New ways of rapidly and easily determining the exact sequence of the chemical groups that constitute a molecule of DNA make it possible to learn the detailed structure of such "cloned" genes. After the structure is known it can be manipulated to produce DNA structures that function more efficiently in the bacterial cell.

In this article we shall first describe some of these techniques in a general way and then tell how we and our colleagues Argiris Efstratiadis, Stephanie Broome, Peter Lomedico and Richard Tizard applied them in our laboratory at Harvard University to copy a rat gene that specifies the hormone insulin, to insert the gene into bacteria and to get the bacteria to manufacture a precursor of insulin. In an exciting application of this technology Charles Weissmann and his colleagues at the University of Zurich recently constructed bacteria that produce human interferon, a potentially useful antiviral protein.

DNA, RNA and Proteins

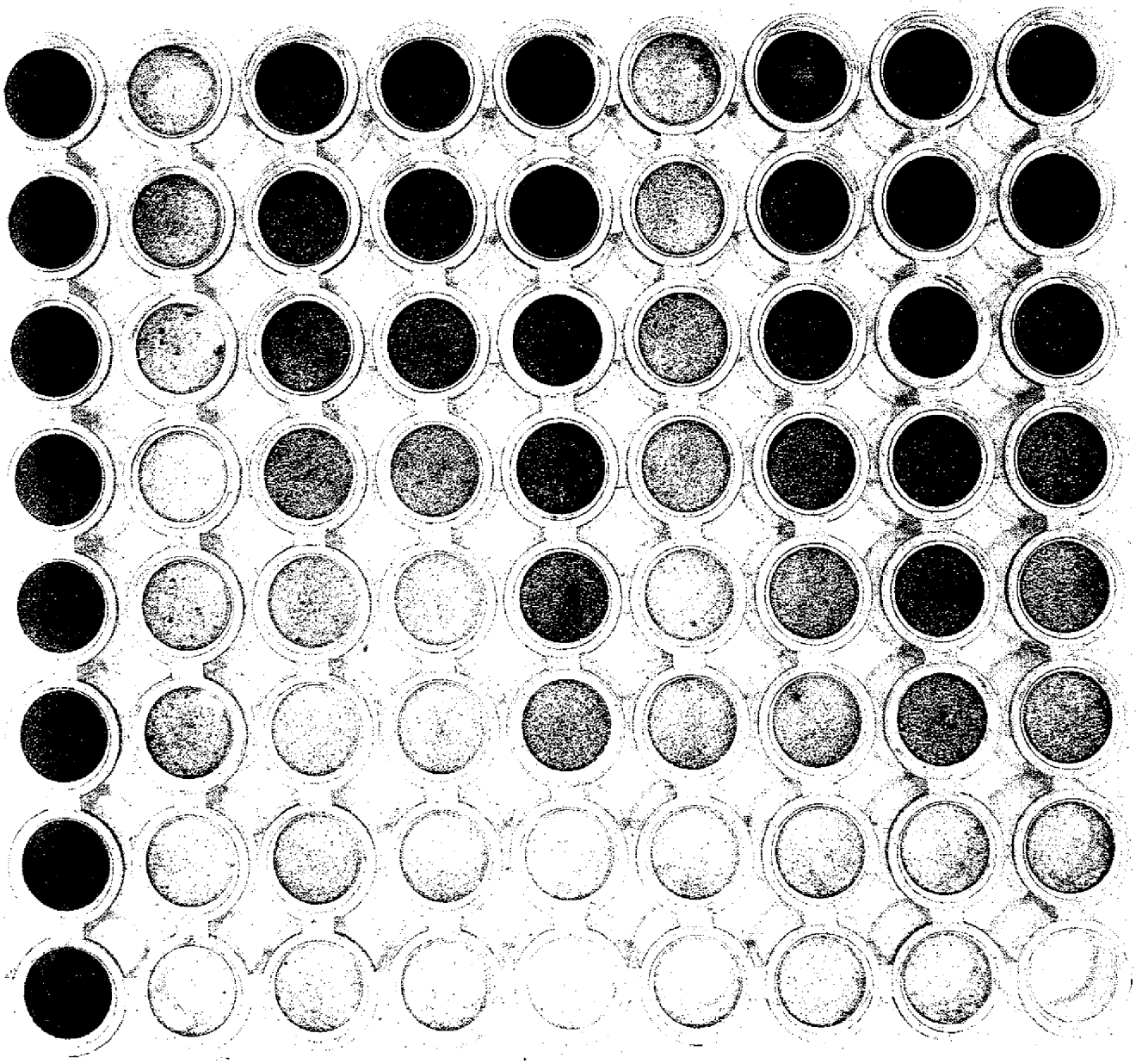
Cells make proteins by translating a set of commands arrayed along a strand of DNA. This hereditary information is held in the order of four chemical groups along the DNA: the bases adenine, thymine, guanine and cytosine. In sets of threes along DNA these bases specify which amino acids, the fundamental building blocks of proteins, are to be used in putting the protein together; the correspondence between specific base triplets and particular amino acids is called the genetic code. The part of a DNA molecule that incorporates the information to specify the structure of a protein is called a structural gene.

To act on this information the cell copies the sequence of bases from its genetic storehouse in DNA into another molecule: messenger RNA. A strand of DNA serves as a template for the assembly of a complementary strand of RNA according to base-pairing rules: adenine always pairs with uracil (which in RNA replaces DNA's thymine) and guanine pairs with cytosine. In animal cells transcription takes place in the nucleus of the cell. The messenger-RNA molecules carry the information out of the nucleus into the cytoplasm, where a complex molecular machine translates it into protein by linking together the appropriate amino acids. In bacteria, which have no nucleus, transcription and translation take place concurrently. The messenger RNA serves as a temporary set of instructions. Which proteins the cell makes depends on which messengers it contains at any given time; to make a different protein the cell makes a new messenger from the appropriate structural gene. The DNA in each cell contains all the information required at any time by any cell of the organism, but each cell "expresses," or translates into protein, only a specific small portion of that information. How does the cell know which structural genes to express?

Along with the structural information, a DNA molecule carries a series of regulatory commands, also written out as a sequence of bases. The simplest of these commands say in effect "Start here" or "Stop here" both for the transcription and for the translation steps. More complicated commands say when and in which type of cell a specific gene should be used. The genetic code is the same in all cell nuclei, a given structural sequence specifying the same protein in every organism, but the special commands are not the same in bacteria and in animal cells. One of the most surprising differences was discovered only in the past two years. The information for a bacterial protein is carried on a contiguous stretch of DNA, but in more complicated organisms, such as pigs and people, the structural information is broken up into segments, which are separated along the gene by long stretches of other DNA called intervening DNA or "introns." In such a cell a long region (often 10 times more than might be needed) is transcribed into RNA. The cell then processes this long RNA molecule, removing the sequence of bases that does not code for the protein and splicing together the rest to make a messenger-RNA molecule that carries essentially just the "start," the structural sequence and the "stop" needed for translation.

To persuade a bacterium to make a nonbacterial protein one must put into bacteria a DNA molecule that has a sequence of bases specifying the protein's amino acids as well as the bacterial commands for transcription and translation. Moreover, the inserted DNA must be treated by the bacterium as its own so that it will be duplicated as the bacterium divides. The problem thus breaks down into three parts: to find the right structural sequence (insulin's, for example), to place it in bacteria in such a way that it will be maintained as the bacteria grow and then to manipulate the surrounding information, modifying the regulatory commands so that the structural sequence is expressed as protein. Once the protein is made, still further changes in its gene or modifications of the bacterium may be needed to obtain the protein in large enough amounts to be useful.

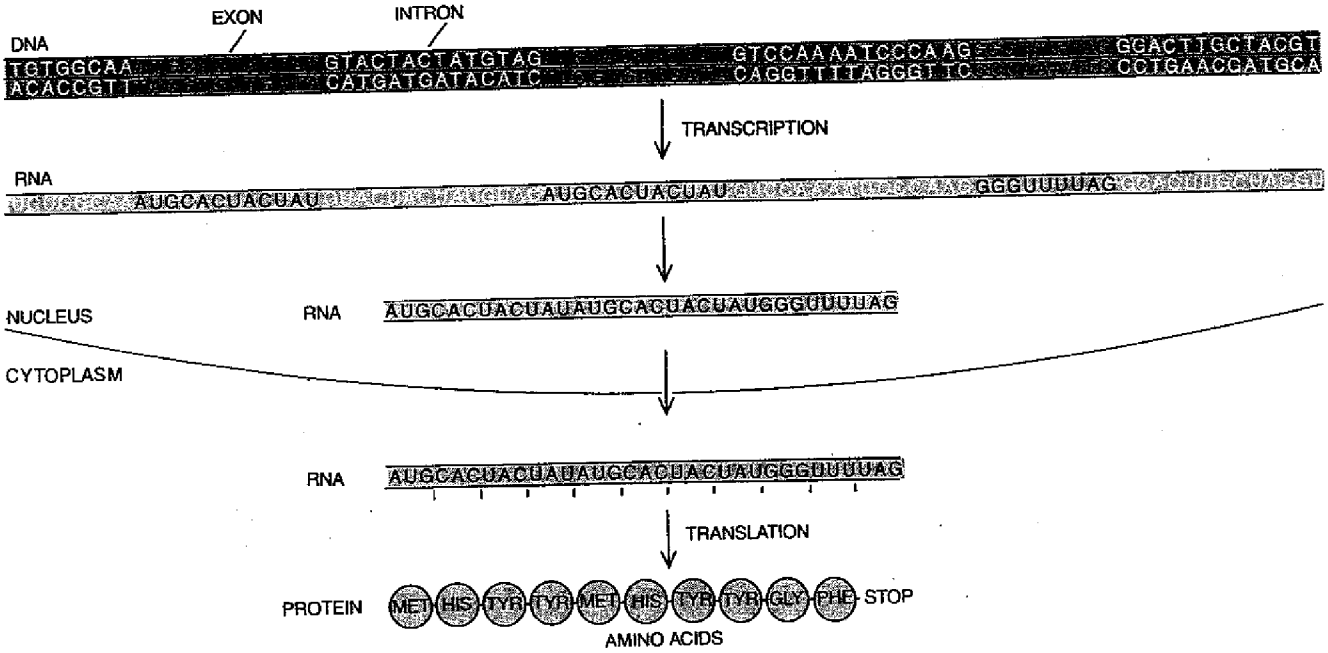
The constellation of recombinant-



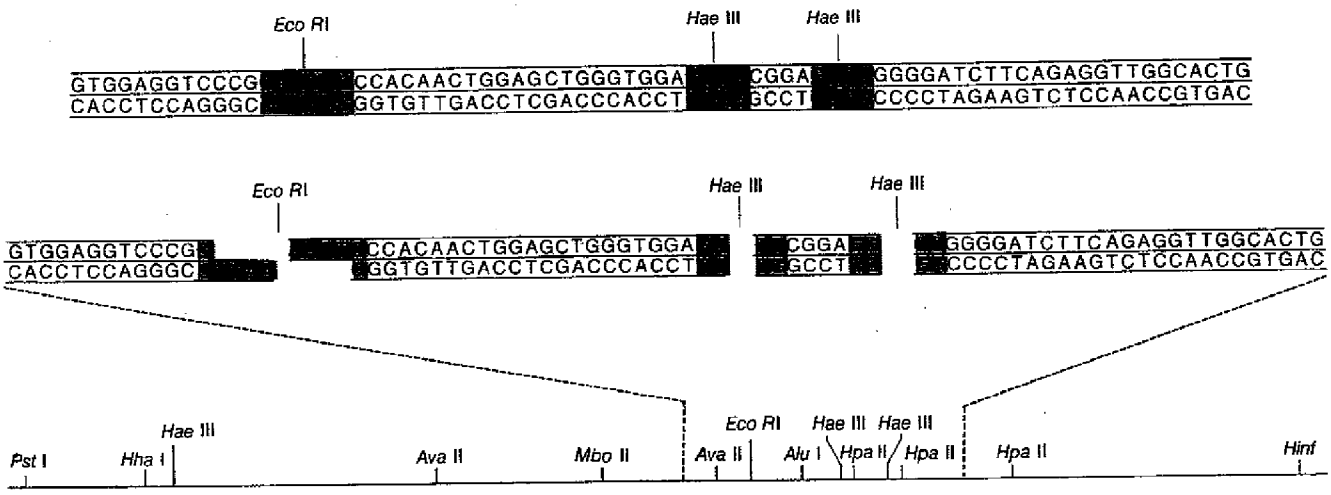
HUMAN INTERFERON synthesized in bacteria demonstrates its ability to block a viral infection in this biological assay. The structural information for making the protein interferon was obtained from human white blood cells in the form of messenger-RNA molecules; the RNA then served as a template for the synthesis of double-strand molecules of copy DNA, and the DNA in turn was inserted by recombinant-DNA techniques into a laboratory strain of the bacterium *Escherichia coli*, which synthesized the protein. For the assay dilutions of an extract of the bacteria were placed in some of the wells of a clear plastic tray; the other wells served as controls. (The wells are seen through the bottom of the tray in this photograph.) Human cells were added to the wells and were grown to form a layer of cells covering the bottom of each well. A virus preparation was then added to the cells. Twenty-four hours later the cell layer was stained. Where interferon in the extracts protected the cells against the virus the cells survived and were stained. Where there was no interferon the virus killed the cells and the dead cells did not pick up the stain. The control wells in the first column at the left contain a layer of cells that were never exposed to the virus; they accordingly appear stained. The control wells in the second column contain cells that have been killed by the virus; they look gray or clear. The control wells in the third column contain dilutions of a standard laboratory sample of interferon obtained directly from human cells; the top well has the most interferon and each succeeding well has a third as much interferon as the well above it. The wells in the next six columns hold dilutions of bacterial extracts from six different colonies of *E. coli* in which interferon DNA was present. Five of the six columns containing the bacterial extracts show evidence of interferon activity. The third extract tested (Column 6) had no detectable interferon; it apparently did not have a complete interferon gene. The synthesis of human interferon by the recombinant-DNA method was achieved by Charles Weissmann and his colleagues at the University of Zurich in collaboration with Kari Cantell of the Finnish Red Cross. The work was supported by Biogen, SA. Interferon is synthesized by many animal cells, but it is species-specific: only human interferon works for human beings, and it has been too scarce even for satisfactory experimentation.

DNA techniques for placing and maintaining a new gene in bacteria is called cloning, which in this sense means the isolation of a specific new DNA sequence in a single organism that proliferates to form a population of identical descendants: a clone. There are two convenient ways of doing this. In one method a small circular piece of DNA called a plasmid is the vehicle for introducing the new DNA into the bacterium. Plasmids carry only a few genes of their own and are maintained in several copies inside the bacterium by the bacterium's own gene functions; they remain separate from the main set of bacterial genes carried on a circle of DNA about 1,000 times larger. Alternatively the vehicle could be a virus that grows in bacteria. Such viruses normally have some 10 to 50 genes of their own (a bacterium has several thousand genes) and can often carry other new DNA segments in place of some of their own. All the techniques we shall describe apply to both plasmids and viruses.

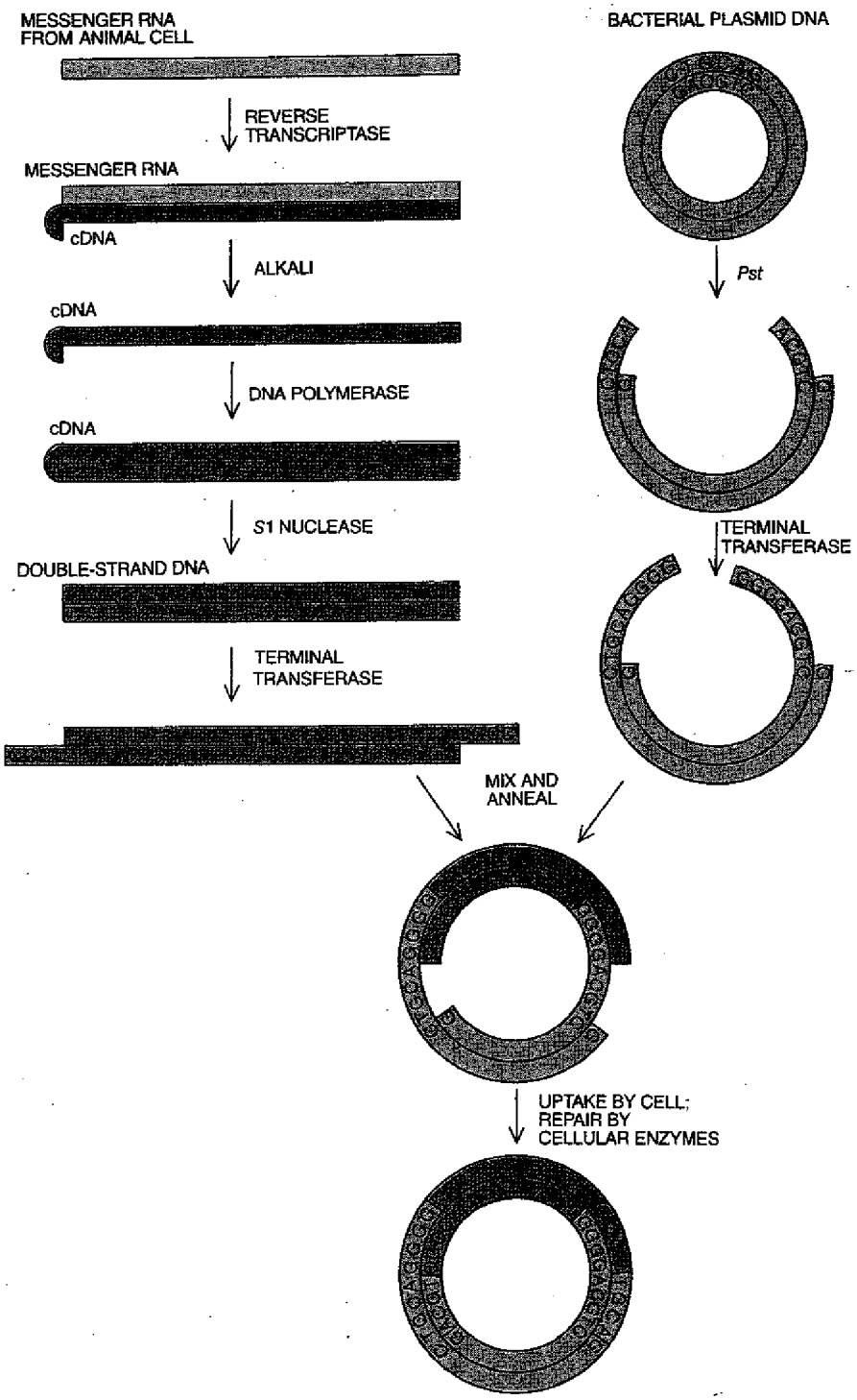
A molecule of DNA resembles a very



PROTEINS ARE MADE in a living cell according to instructions encoded in the cell's genes, which consist of specific sequences of chemical groups (bases) strung out along a double-strand molecule of DNA in the cell's nucleus. The genetic code is "written" in the four letters *A, T, G* and *C*, which stand respectively for the four bases adenine, thymine, guanine and cytosine. The code is "read" in the three-letter sets called codons, which specify the amino acids linked together in the protein chain. The order of the bases can also convey regulatory commands. In multicelled organisms the structural sequence, or gene, encoding a particular protein is usually broken into fragments separated by long stretches of other DNA; in this diagram the gene fragments, called exons, are represented by the black letters and the intervening sequences, known as introns, by the white letters. The genetic information is translated into protein indirectly. First the entire sequence of bases is transcribed inside the nucleus from the DNA to a single-strand molecule of RNA. According to the base-pairing rules governing transcription, adenine always pairs with uracil (*U*) and guanine always pairs with cytosine. Next the RNA copies of the introns are excised from the message and the remaining RNA copies of the exons are joined together end to end. The reassembled strand of messenger RNA then moves from the nucleus to the cytoplasm, where the actual protein-manufacturing process takes place.



DNA CAN BE CUT into comparatively short lengths with the aid of restriction endonucleases, special enzymes that recognize specific base sequences at which they cause the molecule to come apart. For example, *Eco RI*, the first such enzyme discovered, recognizes a certain six-base sequence and cuts the molecule wherever this sequence appears, whereas *Hae* III, another restriction enzyme, operates at a certain four-base sequence. Since the probability of finding a particular four-base sequence is greater than that of finding a particular six-base sequence, one would expect *Hae* III to cut DNA more often than *Eco RI*. Accordingly one *Eco RI* site and two *Hae* III sites are represented in the DNA segment at the top, which corresponds to part of the gene coding for insulin in rat cells. The same DNA contains recognition sites for a number of other restriction enzymes, as is shown in the line diagram of a larger gene fragment at the bottom.



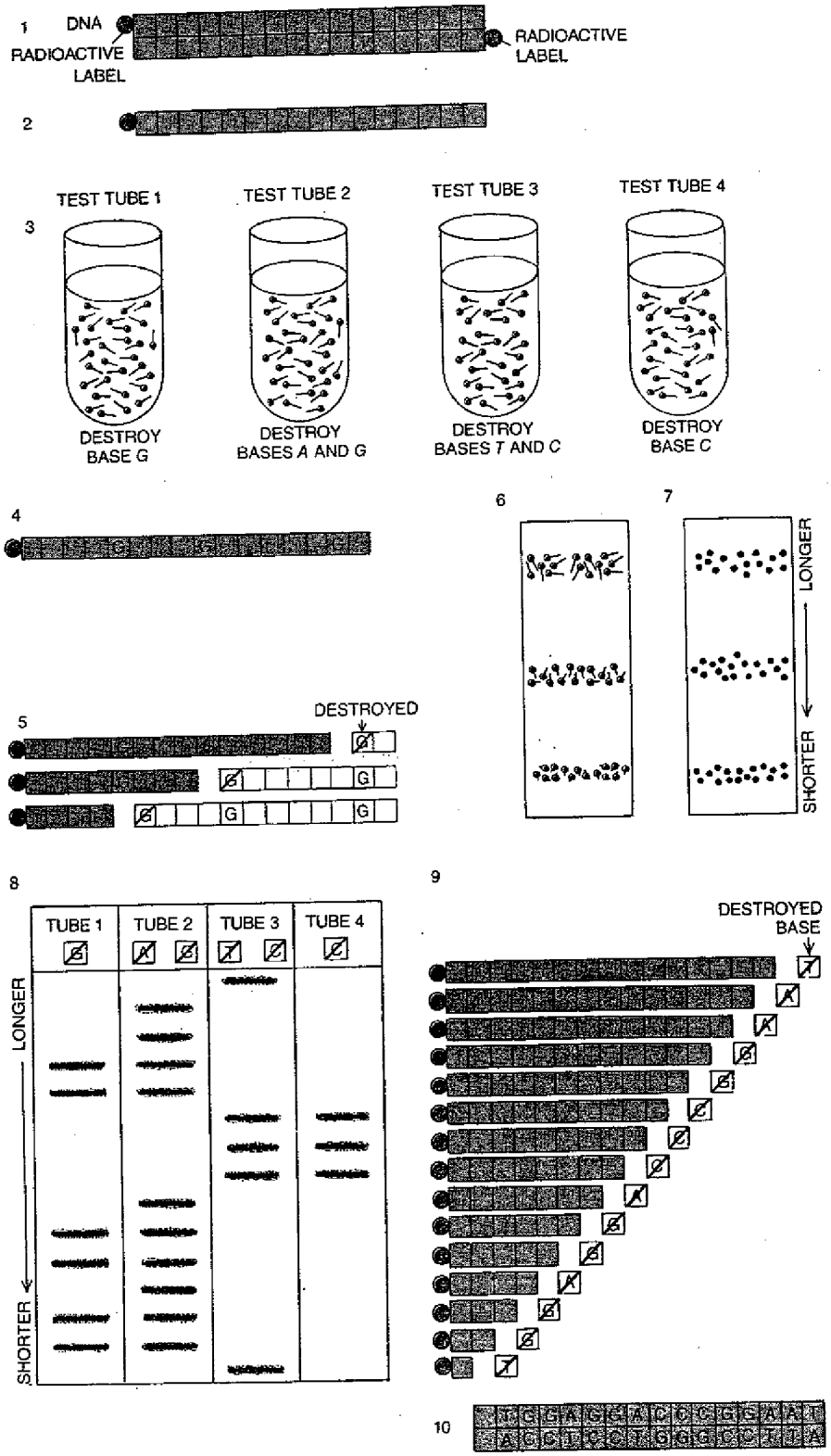
RECOMBINANT-DNA TECHNIQUE for making a protein in bacteria calls for the insertion of a fragment of animal DNA that encodes the protein into a plasmid, a small circular piece of bacterial DNA, which in turn serves as the vehicle for introducing the DNA into the bacterium. The plasmid DNA is cleaved with the appropriate restriction enzyme and the new DNA sequence is inserted into the opening by means of a variety of enzymatic manipulations that connect the new DNA's ends to those of the broken plasmid circle. In the procedure illustrated here, for example, a special enzyme, reverse transcriptase, is first used to copy the genetic information from a single-strand molecule of messenger RNA into a single strand of copy DNA. The RNA template is then destroyed, and a second strand of DNA is made with another enzyme, DNA polymerase. Still another enzyme, *S1* nuclease, serves to break the covalent linkage between the two DNA strands. In the next step the double-strand DNA is joined to the plasmid by first using the enzyme terminal transferase to extend the ends of the DNA with a short sequence of identical bases (in this case four cytosines) and then annealing the DNA to the plasmid DNA, to which a complementary sequence of bases (four guanines) has been added. Bacterial enzymes eventually fill the gaps in the regenerated circular DNA molecule and seal the connection between the inserted DNA and the plasmid DNA. The particular plasmid used by the authors to make rat proinsulin in bacteria, designated *pBR322*, incorporates two genes that confer resistance to two antibiotics: penicillin and tetracycline. The plasmid is cleaved by the restriction enzyme *Pst* at a recognition site that lies in the midst of the gene encoding penicillinase (the enzyme that breaks down penicillin). The added DNA destroys this enzymatic activity, but the tetracycline resistance remains and is used to identify bacteria containing the plasmid.

long, twisted thread. A bacterium has one millimeter of DNA in a continuous string of some three million bases folded back and forth several thousand times into a space less than a micron (a thousandth of a millimeter) across. In human cells the DNA is packed into 46 chromosomes, each one containing about four centimeters in a single piece, the total amount corresponding to about three billion bases. How can one find and work with a single gene only a few thousand bases long? Fortunately nature has devised certain enzymes (proteins that carry out chemical reactions) that solve part of the problem. These special enzymes, called restriction endonucleases, have the ability to scan the long thread of DNA and to recognize particular short sequences as landmarks at which to cut the molecule apart. Some 40 or 50 of these enzymes are known, each of which recognizes different landmarks; each restriction enzyme therefore breaks up any given DNA reproducibly into a characteristic set of short pieces, from a few hundred to a few thousand bases long, which one can isolate by length.

One can clone such DNA pieces in bacteria. As a first step one purifies the circle of plasmid DNA. The sequences of the plasmids are such that one of the restriction enzymes will recognize a unique site on the plasmid and cut the circle open there. One can insert a chosen DNA fragment into the opening by using a variety of enzymatic techniques that connect its ends to those of the circle. Ordinarily this recombinant-DNA molecule could not pass through the bacterial cell wall. A dilute solution of calcium chloride renders the bacteria permeable, however; in a mixture of treated cells and DNA a few bacteria will take up the hybrid plasmid. These cells can be found among all those that did not take up the DNA if a gene on the plasmid provides a property the bacterium must have to survive, such as antibiotic resistance. Then any bacterium carrying the plasmid will be resistant to the antibiotic, whereas all the others will be killed by it. When one spreads the mixture of bacteria out on an agar plate containing nutrients and the antibiotic, each single bacterium with a plasmid will grow into a separate colony of about 100 million cells. A single colony can be chosen and grown further to yield billions of cells, each of which contains identical copies of the new DNA sequence in a recombinant plasmid.

The Sequencing of DNA

The procedures we have outlined so far are followed in "shotgun" cloning experiments. One breaks up the DNA of an animal cell into millions of pieces and inserts each piece into a different bacterium. In this way a number of collections of all the fragments of human,



SEQUENCING OF DNA, in the method devised by one of the authors (Gilbert) and Allan M. Maxam, begins with the attachment of a radioactive label to one end of each strand of double-strand DNA (*1*). The strands of trillions of molecules are separated (*2*) and a preparation of one of the two kinds of strands is divided among four test tubes (*3*). Each tube contains a chemical agent that selectively destroys one or two of the four bases *A, T, G* and *C,* thereby cleaving the strand at the site of those bases; the reaction is controlled so that only some of the strands are cleaved at each of the sites where a given base appears, generating a set of fragments of different sizes. A strand containing three *G*'s (*4*), for example, would produce a mixture of three radioactively labeled molecules (*5*). The reactions break DNA at the *G*'s alone, at the *G*'s and the *A*'s, at the *T*'s and the *C*'s, and at the *C*'s alone. The molecules are separated according to size by electrophoresis on a gel; the shorter the molecule, the farther it migrates down the gel (*6*). The radioactive label produces an image of each group of molecules on an X-ray film (*7*). When four films are placed side by side (*8*), the ladderlike array of bands represents all the successively shorter fragments of the original strand of DNA (*9*). Knowing what base or pair of bases was destroyed to produce each of the fragments, one can start at the bottom and read off a left-to-right sequence of bases (*10*), which in turn yields the sequence of the second strand.

mouse, rat and fly DNA have been made. One can determine the structure of any one of these cloned DNA's by breaking up the hybrid plasmid with a restriction enzyme, separating the resulting DNA fragments, determining the base sequence of each of the fragments and then putting the sequences together to deduce the entire structure of the cloned DNA.

There are two methods for sequencing DNA. Both exploit reference points created by restriction-enzyme cleavage of the DNA at a specific short sequence and then work out the rest of the sequence by measuring the distance of each base from that cut. They do this by creating a set of radioactively labeled molecules, each of which extends from the common point to one of the occurrences of a specific base. When these molecules are separated by size and detected by their radioactivity, the length of the smallest one shows the position of the first occurrence of that base; longer molecules correspond to later occurrences. The pattern created by the analysis of these molecules looks like a ladder. From the positions of the rungs one reads off the lengths. By comparing four such patterns one reads off a sequence.

One technique, devised by Allan M. Maxam and one of us (Gilbert), makes use of chemical reagents that detect the different chemical properties of the bases and break the DNA there. To generate the set of fragments the reactions are done for a short time, so that the molecule is broken only occasionally instead of everywhere the base occurs; different molecules will be broken at different places. Four different sets of reagents are used to generate the four patterns. The radioactive label is attached directly to the end of the particular restriction fragment one wants to sequence, so that only the molecules stretching from the labeled end to the break are detected by their radioactivity.

The other sequencing method, devised by Frederick Sanger of the British Medical Research Council Laboratory of Molecular Biology in Cambridge, makes a DNA copy with an enzyme and stops the sequential synthesis, and hence the elongation of the copy, by blocking the movement of the enzyme at a specific base. Here the radioactive label is incorporated into the newly synthesized molecule in four different reactions. Both methods can provide the sequence of from 200 to 300 bases in a single experiment. One of the small plasmids involved in our cloning experiments was sequenced in a year by Gregory Sutcliffe, who worked out the order of the 4,357 bases on one strand and checked them by working out the complementary strand.

Any DNA region carried on a plasmid can be isolated and sequenced. The difficulty is not in determining the sequence but in obtaining the specific

DNA fragments needed. The recombinant-DNA technique serves almost as a microscope to isolate and to magnify, by making many copies, a DNA region, but one does not want to look through a million bacteria to find a specific gene. The fundamental problem, which has no general solution, is to place only the desired DNA sequence—the desired structural gene—in a bacterium.

Getting the Right Gene

One straightforward approach is suitable for very small proteins. The amino acid sequence and the genetic code will predict a sequence of bases that can specify those amino acids. One can then chemically synthesize a corresponding DNA molecule. Exactly this was done by Keiichi Itakura and his co-workers at the City of Hope National Medical Center in Duarte, Calif., who constructed a DNA sequence 42 bases long that dictates the structure of somatostatin, a small hormone consisting of 14 amino acids. The longer the stretch of DNA, however, the harder it is to make; the synthesis of a stretch of DNA 100 bases long is extremely difficult. Many small hormones consist of from 50 to 100 amino acids, and enzymes and other proteins range from 200 to several thousand amino acids in length. Furthermore, one does not know the amino acid sequence of many interesting proteins. (Indeed, the amino acid sequence of some of these proteins has become available only through the sequencing of cloned DNA.)

The desired structural gene is present, of course, somewhere on the DNA of the animal cell. The problem is to find it, but even if that were possible, the structural information would be broken up (as we mentioned above) by long stretches of other DNA. The information does exist in a continuous form, however, on the messenger RNA. Moreover, different cells specialize in the synthesis of different proteins, so that the appropriate tissue will contain the desired messenger RNA along with other messengers for the common proteins made by all cells. Insulin, for example, is made by the beta cells of the pancreas; those cells contain insulin messenger RNA and other cells do not, even though the insulin gene is present in the DNA of every cell.

The task is then to convert the desired structural information from the cell's messenger RNA into DNA, which can be cloned. For this one takes advantage of a special enzyme, reverse transcriptase, that can copy a single strand of RNA to make a complementary strand of DNA. (The enzyme is found in certain RNA viruses that reverse the normal DNA-to-RNA transcription. Such viruses depend on RNA rather than DNA to carry their information from one cell to another and convert the RNA

back into DNA with the help of reverse transcriptase after they infect a new cell.) One takes this strand of complementary DNA, called copy DNA, and makes a second strand of DNA with the more usual DNA-copying enzyme. The resulting double-strand cDNA fragments are more or less complete copies not only of the desired messenger RNA but also of all the other messenger RNA's that were present in the tissue. At best, however, only a few of the DNA fragments contain all the wanted structural information. Even in those fragments the regulatory signals that surround the structural sequences refer to translation in the animal cell, not in bacteria, and (since the DNA was made from RNA) there will be no transcriptional commands. Although the cDNA can be cloned, two problems remain: to detect any clones containing the sought-after structural DNA fragment and to provide the appropriate signals.

Finding the Right Clone

It is simple to find the right clone if the experiment began with a pure messenger RNA. One can detect matching sequences by the process called hybridization. The two strands of a DNA molecule can be separated by heating, which breaks the weak bonds that hold the two strands together without breaking the strong chemical bonds between bases along the chain. When a mixture of such strands is cooled, those sequences that match will find each other. The first step of this process is called denaturation, the second step reannealing. The same process serves to identify sequence matches between RNA and DNA.

One grows bacterial colonies on a disk of cellulose nitrate paper, breaks open the bacterial cells where they lie and fixes the released DNA to the paper. When the DNA is denatured and reannealed to radioactive RNA, only the remains of those colonies that contained a plasmid whose sequence matches the messenger become radioactive. Since one keeps a replica (a living duplicate set of the colonies), one can obtain bacteria containing the desired DNA. One grows these bacteria to provide material to identify, in further hybridization tests, other clones that contain the same sequence in different surroundings and may turn out to be more effective in producing the wanted protein.

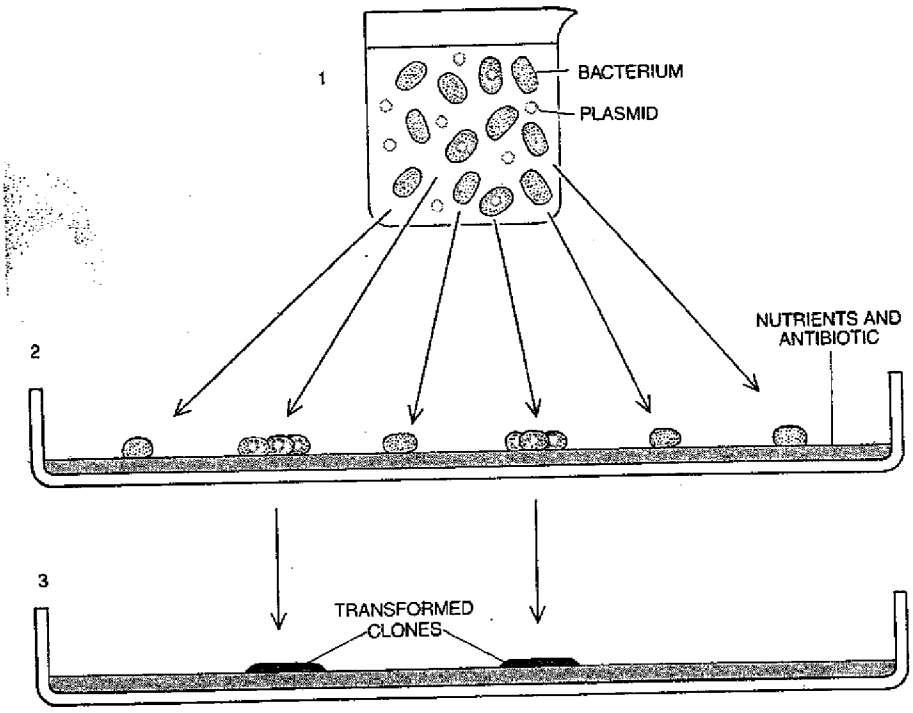
If one cannot purify the messenger RNA because the specific messenger is a small fraction of all the messengers in a cell, there are other ways to search for the DNA sequence. One useful property is the detailed shape of the corresponding protein molecule. Those shapes that are most different and distinctive can be recognized by the protein molecules called antibodies. Animals make antibodies as part of their protective response to foreign substances. If one injects human insulin into a guinea pig, for example, the guinea pig will make antibodies that bind to human insulin. These antibodies will not bind to guinea pig insulin because they "see" only the shapes that make the human protein different. A purified antibody, then, can serve as a reagent to detect a particular protein. (This is the way vaccines work. If an animal is injected with an inactivated virus, it is stimulated to make antibodies against the viral proteins. Thereafter the antibodies will protect the animal against infection by that virus by binding to the virus particle and signaling other cells to remove the invader. Without the earlier stimulation the antibody response to the invading virus is too slow to block the infection.)

Even without purifying a specific messenger RNA one can make the RNA molecules function in the test tube by adding the machinery needed to translate the messengers (obtained from the cytoplasm of broken cells) along with radioactive amino acids. Among the small amounts of radioactive proteins that are synthesized one can recognize the protein of interest with antibodies. This provides a means of detecting the presence of a specific messenger. If one takes a recombinant plasmid and hybridizes it to the mixture of RNA's, only the RNA that matches a sequence in the plasmid will anneal to it and therefore no longer function in translation; the plasmid of interest is detected by its ability to block the synthesis of the desired protein. This identification can be verified because the RNA bound to the DNA can be separated from all the other RNA's and then released from the DNA, whereupon it will function to direct the synthesis of the protein.

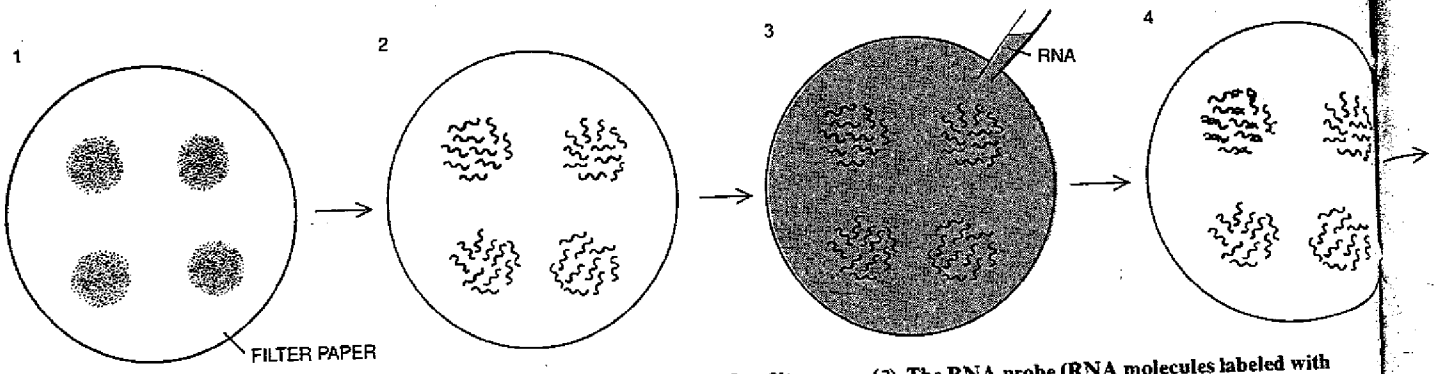
Regulatory Signals

With these techniques one can clone and identify DNA fragments carrying the information that dictates the structure of a protein. Will the information work in bacteria?

One must provide regulatory signals the bacterium can use. One of them is the signal to start the synthesis of a messenger RNA; in bacteria it is a region of DNA immediately in front of the segment of DNA that will be transcribed into RNA. The second important signal functions as part of the messenger RNA, telling the bacterial translation machine to "Start here." All bacterial genes have these two kinds of start signals (some of which work better than others). They also have two stop signals, one for translation and one for transcription. A simple way to make the new protein sequence is to cut a bacterial gene open in its middle with a restriction enzyme and to insert the new DNA there. This results in a hybrid protein that starts out as some bacterial protein and then continues as the string of amino acids one wants. That is how the chemically synthesized gene for somatostatin was made to work in bacteria. The DNA for those 14 amino acids, followed by a stop signal, was inserted near the end of a 1,000-amino-acid protein. After the bacterium made the hybrid protein the



RECOMBINANT PLASMIDS (*color*) bearing the inserted animal-protein genes and genes for resistance to tetracycline are mixed with bacteria (*1*). Some cells take up the plasmid. The mixture of cells is spread on a culture medium containing the antibiotic (*2*), which kills all the cells that do not have the plasmid. The cells that have taken up the plasmid are antibiotic-resistant; they live, and each of them gives rise to a clone, a colony of genetically identical cells (*3*).



CLONE CONTAINING DESIRED DNA can be found among all the successfully transformed clones (*1*) by means of RNA-DNA hybridization if one has a pure messenger-RNA probe for the desired sequence. The cells are broken open and their DNA is denatured and fixed to filter paper (*2*). The RNA probe (RNA molecules labeled with a radioactive isotope) is added (*3*). The RNA (*color*) will anneal to any DNA whose sequence it matches, forming RNA-DNA hybrids (*4*); the remainder of the RNA is washed away. The presence of the hybrid is revealed by the radioactivity.

somatostatin part was cleaved off chemically and purified.

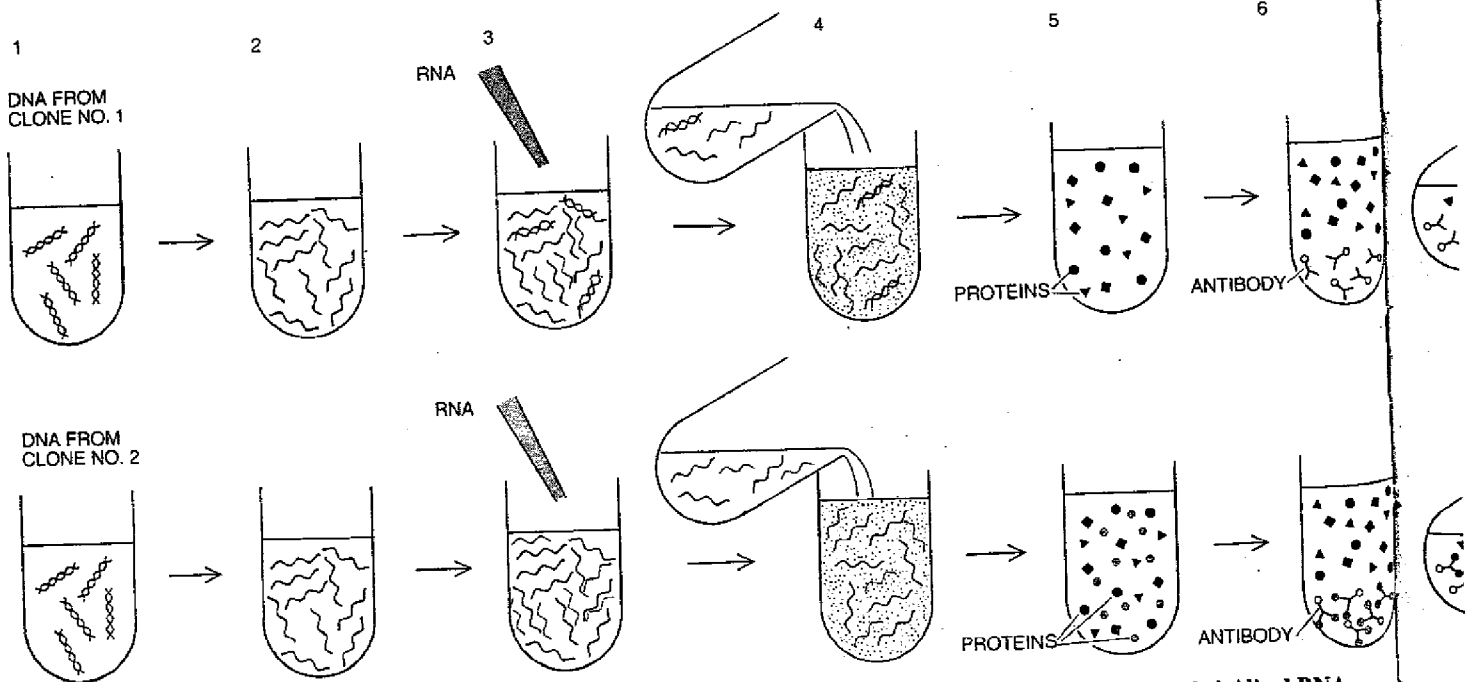
Not only can the bacterial gene serve to provide the regulatory signals but also it may endow the hybrid protein with further useful properties. For example, a few bacterial proteins are secreted through the membrane that surrounds the cell. If one inserts the animal DNA into the gene for such a protein, the bacterial part of the hybrid protein will serve as a carrier to move the new protein through the membrane so that it is more easily observed and purified.

We exploited all the techniques described above to obtain a copy of the insulin gene and to insert it into bacteria to make proinsulin. Insulin is a small hormone made up of two short chains, one chain 20 amino acids long and the other 30 amino acids long. These two chains are initially part of a longer chain of 109 amino acids, called preproinsulin. As preproinsulin is synthesized in the beta cells of the pancreas, the first 23 amino acids of the chain serve as a signal to direct the passage of the molecule through a cell membrane. As this happens those amino acids are cleaved off, leaving a chain of 86 amino acids: proinsulin. The proinsulin chain folds up to bring the first and last segments of the chain together, and the central portion is cut out by enzymes to leave insulin. The role of the central portion is to align the two chains comprising insulin correctly. If the two chains are taken apart later, they do not reassemble easily or efficiently. (In spite of these difficulties Itakura and his co-workers synthesized two DNA fragments corresponding to the two chains of human insulin and attached them separately, like somatostatin, to the same large bacterial gene in order to synthesize two separate hybrid proteins in two different bacteria. Then they cut off the two short pieces, purified them and put them together to form insulin.)

The Proinsulin Experiment

In our experiments we started with a tumor of the insulin-producing beta cells of the rat. (We worked with rat insulin because at the time we began our experiments the guidelines established by the National Institutes of Health for recombinant-DNA investigations would not allow us to insert the human insulin gene into bacteria; that prohibition has since been removed.)

We made DNA copies of the beta-cell messenger RNA and put them into a plasmid, in the middle of a gene for a bacterial protein, penicillinase, that



HYBRID-ARRESTED TRANSLATION, a technique developed by Bryan Roberts of the Harvard Medical School, identifies a clone (*top*) containing the desired DNA even in the absence of a purified RNA probe. DNA from clones being tested (*1*) is denatured (*2*). Unpurified RNA (the same RNA used to make the inserted DNA) is added (*3*); it anneals to any matching DNA. Placed in a "translation system" containing radioactively labeled amino acids (*4*), the unhybridized RNA directs the synthesis of radioactive proteins, but the hybridized RNA cannot be translated; the specific protein (*color*) encoded by the desired DNA is not synthesized in the presence of the clone containing that DNA (*5*). The presence or absence of that protein is determined by an antibody test. Antibody to the protein, fixed to plastic beads,

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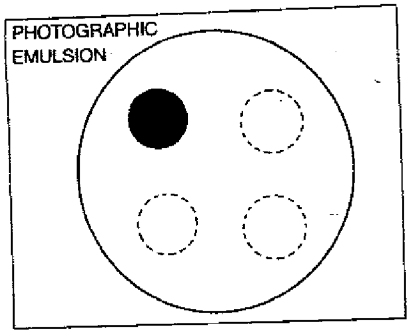

brids is revealed by autoradiography: a photographic emulsion is placed on the filter paper and after exposure the clone containing the desired DNA is identified as a dark spot (5).

would be secreted through the membrane of the bacterial cell. We looked among the bacterial colonies by hybridization, we proved that we had the right hybrid plasmid by blocking the synthesis of insulin in a test tube as we described above and we sequenced the DNA to see exactly what part of the insulin gene we had. Once we had found one hybrid plasmid, we used it to find 48 more by repeating the hybridization test. These 48 clones represented 2 percent of all the clones we had made.

Would any of those clones actually synthesize insulin? We looked among the clones containing insulin DNA for any that were synthesizing a hybrid protein part of which was proinsulin. For this we relied on a sensitive radioactive-antibody test. We coated plastic disks with antibody directed against either insulin or penicillinase and exposed them

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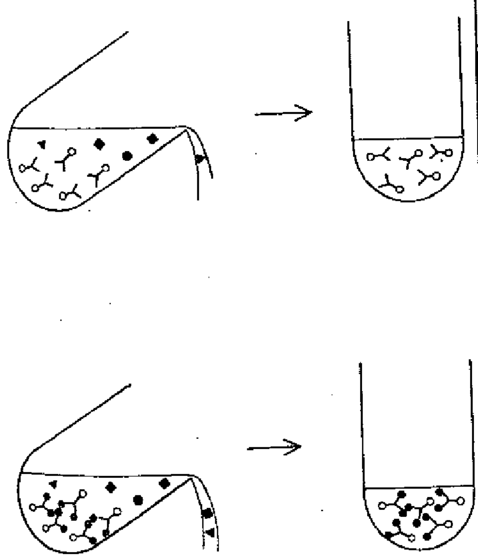
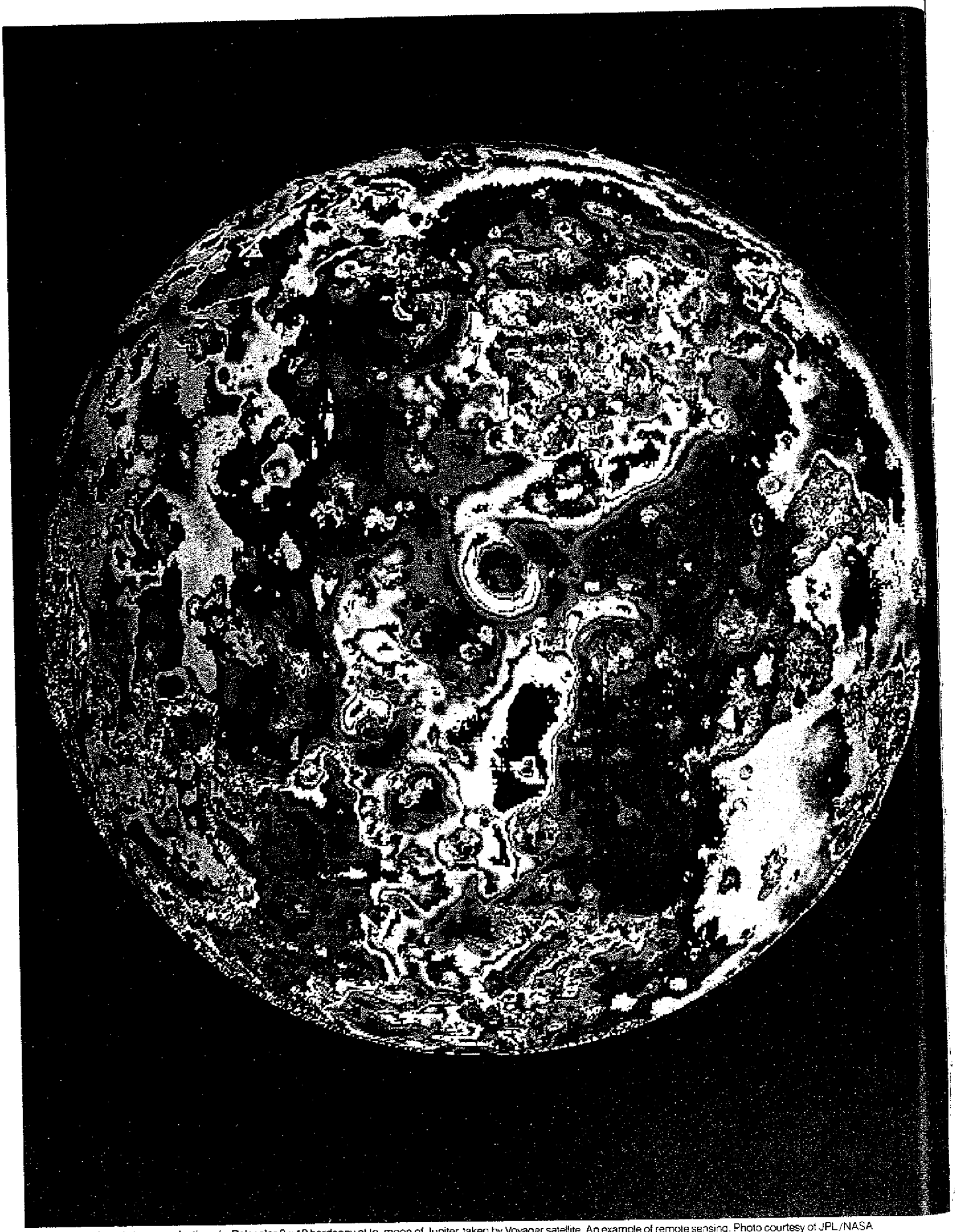


is added and binds the protein, precipitating the protein out of the solution (*6*), which is poured off (*7*). Measurement of the precipitates' radioactivity (*8*) shows that one clone (*top*) contains the desired DNA, because it blocked the synthesis of the specific protein.



Actual-size reproduction of a Polacolor 8 x 10 hardcopy of Io, moon of Jupiter, taken by Voyager satellite. An example of remote sensing. Photo courtesy of JPL/NASA.
All photographs taken with a Dunn Instruments 631 color camera.

Typic

to the contents of cells from each clone. Any insulin (or penicillinase) present in the cells binds to the antibody and is thereby fixed to the plastic disks. Then we applied radioactively labeled anti-insulin antibody to detect the presence of proteins with insulin shapes. One clone gave positive responses, both on disks coated with anti-insulin and on those coated with antipenicillinase, to radioactive antibody to insulin, thereby demonstrating the presence of a penicillinase-insulin hybrid protein.

To see if the bacteria were secreting the hybrid protein we grew the clone in liquid culture and tried to extract the protein by a method that does not burst the bacterial cell membrane. The test showed the fused protein to be present outside the membrane: it was secreted, as we had hoped it would be.

Sequencing the DNA showed that the DNA fragment and the details of the fusion were such that the structural information in the clone was only for proinsulin and did not contain the "pre" region. In order to make insulin we removed most of the bacterial protein and the middle segment of the proinsulin with the digestive enzyme trypsin. Would the insulin made from the bacteria be an active hormone? Stephen P. Naber and William L. Chick of the Elliot P. Joslin Research Laboratory in Boston tested the molecule by showing that it affected the metabolism of sugar by fat cells, as it should.

Improving the Yield

The amount of proinsulin made by the original clone was very small; we are currently engaged in various manipulations to improve the yield. Regulatory signals must be not only efficient but also optimally placed. One need not be satisfied with the signals that happen to surround preexisting bacterial genes. With restriction enzymes one can clip out small DNA fragments that carry only the regulatory signals and tie them together with a DNA-linking enzyme to make new combinations. One can trim back the ends of these fragments by nibbling off bases with still other enzymes before reconnecting them. This will alter the spacings between the signals and the structural sequence. Although each of these manipulations generates only a small number of correct molecules, by cloning after each step one can make large amounts of the DNA and work out its sequence, and then continue the tinkering.

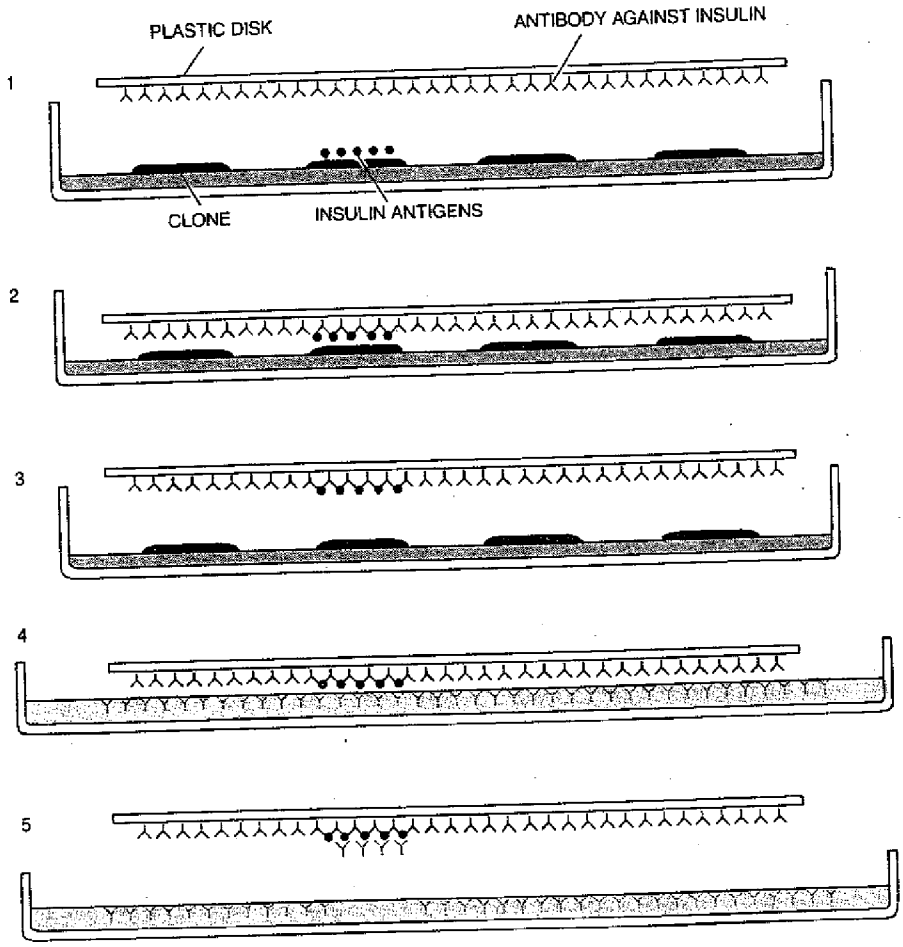
Moreover, one can synthesize short desired DNA sequences and tie them to other fragments. For example, David V. Goeddel and his co-workers at Genentech, Inc., took a piece of DNA containing the structural information for human growth hormone (168 amino acids), connected it to a synthetic piece of DNA containing part of the translational start signal and attached that combination in turn to a fragment containing the rest of the regulatory signals. When this DNA construction was cloned, the bacteria made a protein of the shape (as recognized by antibodies) and size of growth hormone (although not yet with demonstrated hormone activity).

Although we do not yet know the optimal combinations of the DNA elements for making insulin in bacteria, finding them is only a matter of time. There are other problems to be considered. Often the new animal proteins are broken down in the bacterial cell because their structure is such that enzymes normally present in the bacteria can digest them. Ways have to be found to stabilize the proteins either by removing these enzymes, by embedding the new protein in a hybrid protein to protect it or by secreting it from the cell. Messenger-RNA molecules themselves are often unstable within the cell; modifications in their structure and in the cell itself can make them more effective and lead to increased protein synthesis. And if the number of copies of the plasmid carrying the gene in each cell can be increased, more of the product will be made.

While we work to improve the yield of rat proinsulin and to purify it we expect to apply the same methods to the bacterial synthesis of human insulin. Investigators in other laboratories are also working on the problem, and one can hope that eventually the manufacture of human insulin by bacteria will be cheaper than the purification of insulin from pigs and cattle, the present sources of the hormone. Clearly other human hormones can also be prepared by these procedures. What other therapeutic proteins might be made in bacteria? In general any human protein that cannot be obtained in useful form from animals is an excellent prospect.

Other Proteins from Bacteria

Many genetic diseases are caused by the lack of a single protein. Replacement therapy may be possible if such proteins can be made in bacteria. Vaccines against viral or parasitic infections are a further wide class of possibilities. Today in order to make a vaccine one



RADIOACTIVE-ANTIBODY TEST, developed by Stephanie Broome and one of the authors (Gilbert), is used to search among the bacterial clones containing insulin DNA for signs that insulin is indeed being synthesized. A plastic disk coated with an anti-insulin antibody is first exposed to the contents of cells from each clone (*1*). Any insulin present in the cells is bound to the antibody (*2*) and thereby fixed to the plastic disk (*3*). Radioactively labeled antibody (*color*) to insulin is then applied to the disk in order to detect the presence of the protein (*4, 5*). When the test is repeated with a plastic disk coated with an antipenicillinase antibody, only a hybrid protein, part penicillinase and part insulin, will bind the labeled antibody.

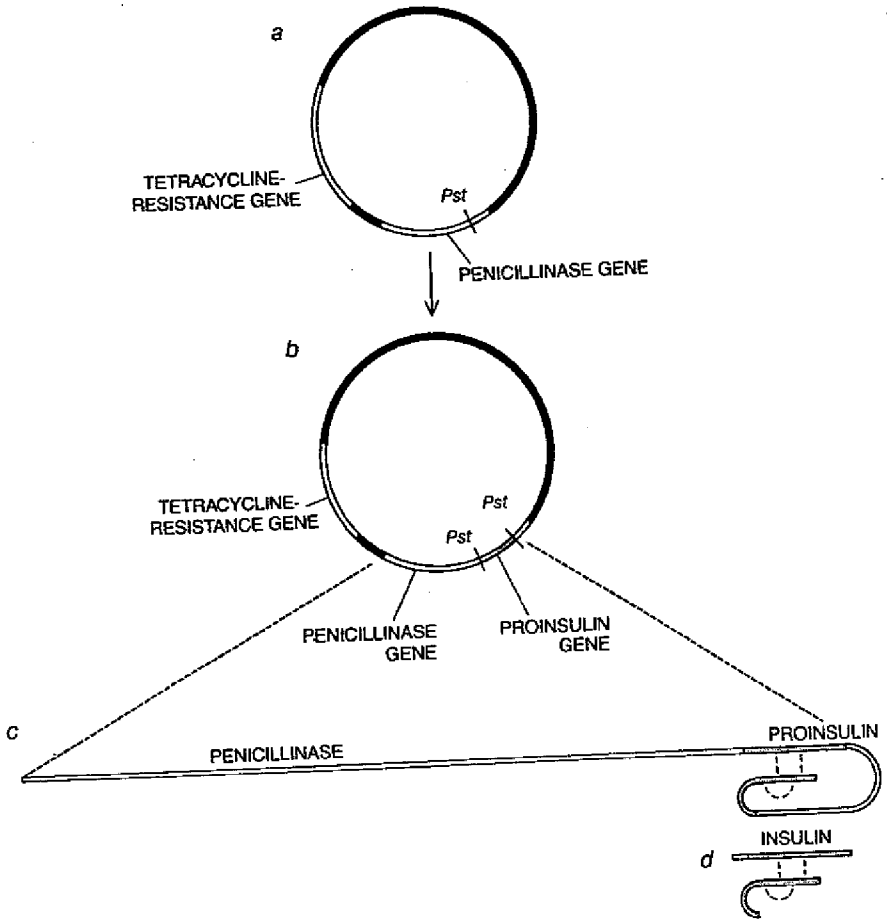
must be able to grow the disease organism in large amounts; often this is impossible or dangerous. Furthermore, the vaccine must be rendered harmless before it is administered, which can be difficult. The new technology offers the chance to make in bacteria only the protein against which the antibody response needs to be directed. This would eliminate any need to work with the intact disease organism. For example, the hepatitis *B* virus, which causes serum hepatitis, cannot be grown outside the body. The only source of this small DNA virus is the blood of infected human beings. The DNA of the virus has now been cloned in several laboratories and its complete sequence has been worked out, revealing the structure of the viral proteins; now the proteins are being made in bacteria. A flood of new information has resulted from this work.

A particularly promising candidate is interferon, a protein cells make to block viral infections quickly. (The antibody response is much slower.) Interferon appears to be the body's first line of defense against viruses. It may also have a therapeutic effect in some cancers. Interferon has never been available in sufficiently large amounts, however, to determine how effective it might really be in protecting against disease. The ability to test the activities of human interferon will soon be a reality because the protein has now been made in bacteria. Weissmann, with his colleagues Shigekazu Nagata, Hideharu Taira, Alan Hall, Lorraine Johnsrud, Michel Streuli, Josef Ecsödi and Werner Boll, along with Kari Cantell of the Finnish Red Cross, applied many of the techniques we have described to clone and to express this protein. The problem they faced was that the messenger RNA for interferon is far rarer than the one for insulin, even in white blood cells that have been stimulated by infection with a virus to make interferon. They took messenger RNA from these white blood cells (17 liters at a time), made double-strand cDNA and cloned it by the procedures we have described.

They looked through some 20,000 clones (in batches) by hybridizing the plasmid DNA from the clones to the messenger RNA of the white blood cells, isolating the RNA that annealed and checking the RNA to see if it was able to direct the synthesis of interferon (not in the test tube but by injecting the RNA into a particularly large cell, a frog's egg). Fortunately interferon is a remarkably potent substance, and so the amount synthesized in the frog's egg could be detected by its ability to protect cells against viruses.

Once Weissmann and his colleagues had found a batch of clones that could hybridize to interferon messenger RNA they tested progressively smaller groups of those clones to find the correct one. Then, with that clone as a probe, they found other clones by means of hybridization testing. Finally they tested extracts of the bacteria carrying the interferon DNA (inserted into the penicillinase gene) directly to see if any of the bacterial clones made biologically active interferon. A number of clones did, confirming that the interferon structural DNA had been correctly identified. The sequencing of the DNA of those clones will determine the structure of interferon, which is still not known.

The amount of interferon made in the bacteria was extremely small: only one or two molecules per cell. (Bacterial proteins are usually made in from 1,000 to 100,000 copies per cell.) We are confident that the methods we have described will solve this problem and lead to the production of enough interferon for clinical tests.



RAT INSULIN WAS OBTAINED by the authors from a hybrid protein composed of part of the bacterial penicillinase molecule and a molecule of proinsulin, an insulin precursor. The map of the plasmid that served as a vehicle, *pBR322* (*a*), shows the location of the genes for the two enzymes conferring antibiotic resistance and the site of cleavage by the restriction enzyme *Pst*. The next map (*b*) shows the structure, as determined by DNA sequencing, of the recombinant plasmid in the bacterial clone that synthesized proinsulin. The proinsulin sequence (*color*) lies between two *Pst* sites that were regenerated in the insertion process. The hybrid protein synthesized by the clone (*c*) comprises most of the penicillinase and also the proinsulin molecule (*color*); broken lines represent disulfide bonds. The authors cut away most of the penicillinase and the middle segment of the proinsulin (*light color*) to make biologically active insulin (*d*).

The Recombinant-DNA Debate

The development of the genetic-engineering techniques described in this article was greeted, over the past decade, with both excitement and alarm. The possible benefits of the techniques were obvious, but some people felt there was reason for concern. Biologists called for an evaluation of the possible hazards of this research; the result was an unprecedented national and international effort in which the public, governments and the scientific community joined to monitor research activities. New knowledge about the properties of genes and the behavior of the bacteria used in this work (usually *Escherichia coli*) has led to a steady lessening of these concerns and to a relaxation of the guidelines that once restricted such experiments. In retrospect, with the advantage of hindsight, the concerns about hypothetical hazards seem to have been unwarranted. We know of no adverse effects from this research. The great potential of the new techniques, both in promoting the growth of basic knowledge and in making possible the synthesis of products of direct benefit to society, is much closer to realization than seemed likely only a few years ago.

TAB G

90. Construction and Identification of a Bacterial Plasmid Containing the Human Fibroblast Interferon Gene Sequence

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Introduction. Interferons are species specific glycoproteins (Weil and Dorner, 1973) which are produced by various cells upon induction with viruses and double-stranded RNAs as well as other compounds, imparting an antiviral state in the recipient cells (Isaacs and Lindenmann, 1957). Beside their antiviral function, other multiple functions such as antitumor and anticellular functions have been described (see The Interferon System, 1977 for reviews). Interferons produced in cultures of human buffy coat cells and in human fibroblasts exhibit a number of distinct properties (Havell *et al.*, 1975; Berg *et al.*, 1975; Gresser *et al.*, 1974; Vilcek *et al.*, 1977), suggesting that leucocyte interferon and fibroblast interferon are different proteins coded for by distinct structural genes (Cavalieri *et al.*, 1977).

A number of attempts have been made for physical and chemical characterization of the interferon molecule and for its clinical investigation. However, mainly due to the low amount of this protein produced by the cell, it is as yet difficult to obtain sufficient quantities of purified interferon for these studies.

One approach to solve the problem appears to be the use of recombinant DNA technology. As has been the case for many proteins, cloning of the gene coding for interferon will provide a definitive answer to the molecular structure of this protein as well as the organization of the gene. In addition, with the aid of gene manipulation, cloned DNA may serve as a potent interferon producer in bacterial or in cognate host. Here, we present evidence for successful construction of a hybrid plasmid DNA containing a human fibroblast interferon gene sequence.

Materials and methods. Human foreskin fibroblast strain DIP2, a high interferon producer, has been isolated by us. Superinduction

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of the cells by Poly (I) : (C) and cycloheximide, and isolation of total cytoplasmic mRNA were carried out essentially as described (Sehgal *et al.*, 1978). Synthesis of double-stranded cDNA (Maniatis *et al.*, 1976), transformation of *Escherichia coli* χ 1776 by pBR322-cDNA hybrid (Enea *et al.*, 1975) and detection of the specific recombinant DNA by *in situ* colony hybridization (Grunstein and Hogness, 1975) were performed essentially according to the published procedures. Preparation of plasmid DNA was according to the procedure of Currier and Nester (1976) with some modifications. Trapping of the mRNA-DNA hybrid on nitrocellulose filter (Nygaard and Hall, 1963) and elution of the mRNA from the filter (Harpold *et al.*, 1978) were carried out essentially as described. Microinjection of mRNA into *Xenopus* oocytes was performed according to Gurdon *et al.* (1971). The interferon activity was determined following the procedure of Suzuki *et al.* (1974).

Results and discussion. Total cytoplasmic RNA was extracted from human fibroblast DIP2 cells after four hours induction by poly (I) : (C) in the presence of cycloheximide and poly(A) containing mRNA was isolated by oligo(dT)-cellulose affinity chromatography. Starting from 1.5×10^9 cells, 250 μg of poly(A) containing mRNA was obtained. After fractionation of the mRNA with a 5%–25% sucrose gradient centrifugation into 20 fractions, small portion of the mRNA from the fractions around 12S region was injected into *Xenopus* oocytes and interferon activity was determined as described in Materials and methods.

Table I. Interferon mRNA activity in the fractions from sucrose gradient

Fraction No.	Interferon activity (units/ml)
9	<50
10	44
11	550
12	52

RNA from sucrose gradient (input 250 μg) was precipitated, dissolved in 20 μl H_2O and an aliquot (about 0.2 μl) was injected into *Xenopus* oocytes as described (Gurdon *et al.*, 1971). Interferon activity was measured according to Suzuki *et al.* (1974).

As shown in Table I, the highest interferon mRNA activity was detected in fraction 11. This fraction contained about 5 μg of mRNA (termed "interferon mRNA" here), which was used as the template for cDNA synthesis. About 1.5 μg of double-stranded cDNA was synthesized, elongated with dAMP residues, then hybridized with EcoRI-cleaved, dTMP-elongated pBR322. This hybrid DNA was used

to transform *E. coli* strain χ 1776 in the P3 laboratory of Cancer Institute and colonies containing the hybrid plasmid DNA were selected on agar plates containing ampicillin (20 μg/ml). Efficiency of the transformation was about 1.5×10^5 colony/μg cDNA. For the first screening, 3600 colonies were picked up and transferred on grid-meshed nitrocellulose filters in triplicate. After the colonies had grown on the filters, DNA from duplicate filters was fixed for *in situ* colony hybridization (Grunstein and Hogness, 1975). Colonies grown on the third filter were kept at 4°C.

For colony hybridization, two kinds of ^{32}P-labeled cDNA probe were prepared as follows. First, partially purified "interferon mRNA" (6 μg) was prepared as described above and ^{32}P-labeled cDNA synthesized (0.45 μg, specific radio activity: 6×10^8 c.p.m./ μg). After removing the template RNA by alkali treatment, cDNA was hybridized with about 50 fold excess of mRNA prepared from mock-induced cells incubated for four hours in the presence of cycloheximide. Non-hybridized cDNA consisting of about 10% of the total radioactivity was separated from mRNA-cDNA hybrid by hydroxyapatite column chromatography and this cDNA was used as probe A. After alkali treatment to remove mRNA, cDNA which had hybridized with mRNA was used as probe B. Both probe A and probe B were separately hybridized with colony DNA fixed on nitrocellulose filters (Grunstein and Hogness, 1975). Colonies hybridized with probe A but failed to hybridize or hybridized much less with probe B were screened by autoradiogram. Four colonies, nos. 319, 644, 746 and 3578, were in this category (Table II).

Table II. Colony hybridization of the cDNA clones with different cDNA probes

Ampicillin resistant colony	Extent of hybridization	
	probe A	probe B
no. 319	++++	++
no. 644	+++	+
no. 746	++	−
no. 3578	+++++	+

Colony hybridization was done according to Grunstein and Hogness (1975). Extent of hybridization was determined by visual inspection of the autoradiogram.

For the second screening, these four colonies were grown in liquid media and each recombinant plasmid DNA was prepared as described in Materials and methods. 5 μg of each recombinant plasmid or pBR322 DNA was linealized by Hind III digestion, denatured, and then hybridized with the "interferon mRNA" (2.5 μg) under the con-

ditions where RNA-DNA hybrid could be formed whereas DNA renaturation was negligible (Casey and Davidson, 1977; details to be published elsewhere). After 4 hrs of hybridization, single-stranded plasmid DNA whose cDNA part should have been hybridized with complementary mRNA was trapped on nitrocellulose filter (Nygaard and Hall, 1963) and hybridized mRNA was eluted from the filter (Harpold *et al.*, 1978). After oligo (dT)-cellulose column chromatography to remove possible contaminants such as DNA, mRNA was microinjected into frog oocytes to determine the interferon mRNA activity. As shown in Table III, mRNA hybridized with the recombinant plasmid DNA from clone no. 319 gave rise to interferon synthesis whereas mRNA hybridized with the other plasmid DNA including pBR322 failed to synthesize interferon in frog oocytes. When the same amount of "interferon mRNA" as that used for hybridization was incubated as a control under the same conditions without DNA, chromatographed on the oligo(dT)-cellulose column and injected into oocytes, approximately 1000 interferon units per ml equivalent was synthesized. Since about 50% of the DNA (i.e. mRNA-DNA hybrid) can be trapped on the filter under our conditions, interferon mRNA must have hybridized with clone no. 319 DNA at an efficiency of about 72% of the input or even higher (Table III).

Table III. Hybridization-translation assay with interferon mRNA and various recombinant DNA

Plasmid DNA	Interferon activity (units/ml)
from no. 319	360
from no. 644	<10
from no. 746	15
from no. 3578	<10
pBR322	<10

"Interferon mRNA" was hybridized with linealized, denatured plasmid DNA and the hybrid was trapped on nitrocellulose filter. RNA was eluted, chromatographed on an oligo (dT)-cellulose column, then injected into frog oocytes for interferon synthesis. Interferon activity was measured as described in Meterials and methods.

In the next experiment, double amount of the mRNA and plasmid DNA from clone no. 319 as those used in the previous experiment was mixed in the hybridization solution and a half portion of the mixture was immediately withdrawn for filter trapping (zero time) and the rest was allowed to hybridize for 4 hrs. Interferon mRNA activity in the RNA trapped on the filter was determined as described above. The interferon activity at zero time and after 4 hrs incubation was

<30 units/ml and 150 units/ml, respectively.

These results thus indicate that the recombinant plasmid DNA from clone no. 319, named TpIF319, contains the sequence for the interferon mRNA. We have also observed that when TpIF319 DNA was hybridized with a mixture of "interferon mRNA" and rabbit globin mRNA, interferon mRNA activity but not globin mRNA activity was detectable from the filter-trapped RNA, whereas an opposite result was obtained when TpIF319 DNA was replaced with pBR322 DNA containing rabbit β-globin gene insert. In addition, both mRNAs were trapped on the filter when two of the recombinant plasmid DNA were present in the hybridization mixture (to be published elsewhere).

In conclusion, we have repotred in this paper the construction of the recombinant plasmid DNA which contains human fibroblast interferon gene sequence. The method for the identification of the specific recombinant which we employed in this paper should be applicable for the cloning of any kind of gene which is expressed in even smaller amount in the cell, provided that the mRNA translation product can be identified. The recombinant plasmid we have constructed will be useful not only for defining the hitherto unknown structure of the interferon gene but also for the attempts of the mass production of this protein.

Acknowledgement. We wish to express our sincere gratitude to Dr. H. Sugano, Director of Cancer Institute, for his continuous support and encouragement. Thanks are also due to Drs. Y. Mishima, R. Kominami and H. Koyama, Cancer Institute, for valuable suggestions and discussions. Skilfull technical assistance of Ms. M. Ohkawa is also acknowledged.

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TAB H

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Biochemistry

Functional expression of cloned yeast DNA in *Escherichia coli*

(plasmid ColE1/leucine operon/histidine operon/eukaryote DNA/complementation)

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ABSTRACT **A collection of hybrid circular DNAs was constructed *in vitro* using the poly(dA-dT) "connector" method; each hybrid circle contained one molecule of poly(dT)-tailed DNA of plasmid ColE1 (made linear by digestion with *Eco*RI endonuclease) annealed to a poly(dA)-tailed fragment of yeast (*Saccharomyces cerevisiae*) DNA, produced originally by shearing total yeast DNA to an average size of 8×10^6 daltons. This DNA preparation was used to transform *E. coli* cells, selecting colicin-E1-resistant clones that contain hybrid ColE1–yeast DNA plasmids. Sufficient numbers of transformant clones were obtained to ensure that the hybrid plasmid population was representative of the entire yeast genome. Various hybrid ColE1–yeast DNA plasmids capable of complementing *E. coli* auxotrophic mutations were selected from this population. Plasmid pYe*leu*10 complements several different point or deletion mutations in the *E. coli* or *S. typhimurium leuB* gene (β-isopropylmalate dehydrogenase); plasmids pYe*leu*11, pYe*leu*12, and pYe*leu*17 are specific suppressors of the *leuB*6 mutation in *E. coli* C600. Plasmid pYe*his*2 complements a deletion in the *E. coli hisB* gene (imidazole glycerol phosphate dehydratase). Complementation of bacterial mutations by yeast DNA segments does not appear to be a rare phenomenon.**

A key question invoked by the recent discovery of methods to biochemically construct recombinant DNAs concerns the ability of fragments of eukaryotic DNA inserted into plasmid or phage vectors to be expressed meaningfully in a bacterium such as *Escherichia coli*. In order for expression to occur, components of the bacterial cell must transcribe the sense strand of the foreign DNA into mRNA and translate this mRNA into functional protein. In addition, further post-translational modifications may also be required to obtain an active product. Major differences apparently exist between eukaryotes and prokaryotes in several of these vital components and processes, such as the RNA polymerases, ribosomal subunits, translational initiation requirements, and post-transcriptional and post-translational modifications.

Several laboratories have shown that transcription of cloned eukaryotic DNAs in *E. coli* can occur in a bacterial cell; however, there is no evidence that normal transcription start and stop signals are recognized in the bacterial system (1–3). However, Struhl *et al.* (4) have recently shown that a segment of yeast DNA cloned on a phage vector can complement *hisB* mutations in *E. coli*, a strong indication that a functional gene product is being formed in this heterologous system.

These considerations have also led us to use a sensitive assay for the expression of cloned eukaryotic DNA, the ability to suppress or complement bacterial auxotrophic mutations *in vivo*. Thus, it should be possible to detect a relatively low level of expression of a cloned eukaryotic gene by the relief of a metabolite requirement in a mutant bacterial strain. In addition, a wide variety of *E. coli* strains containing mutations in known genes involved in amino acid and nucleotide biosynthesis are readily available, so that the generality of any complementation phenomena could be established. We also have selected DNA from an organism (*Saccharomyces cerevisiae*) that has many biosynthetic pathways in common with *E. coli*. Because the size of the yeast genome is only 2–3 times that of *E. coli* (5), the number of different recombinant plasmids that we would need to screen in order to cover the entire genome would not be excessively large (6).

Using the poly(dA-dT). "connector" method (7, 8) to join randomly sheared yeast DNA segments to plasmid ColE1 DNA (L_{RI}), we have isolated and characterized ColE1–yeast DNA recombinant plasmids that can complement *leuB* and *hisB* mutations in *E. coli*. The frequency with which the interspecies complementation is observed suggests that it is not a rare phenomenon.

Abbreviations: Leu$^+$, leucine-independent; His$^+$, histidine-independent.

METHODS

Bacterial Strains. The recipient for transformations with poly(dT)-tailed ColE1 DNA (L_{RI}, indicating linear, generated by digestion with endonuclease *Eco*RI) annealed to a collection of poly(dA)-tailed sheared yeast DNA was strain JA199 (*hsdM*$^+$ *hsdR*$^-$ *lacY*$^-$ *leuB6* Δ*trpE5*/F$^+$). It was constructed by transducing strain MV10 (*hsdM*$^+$ *hsdR*$^+$ *lacY*$^-$ *leuB6 thr-1* Δ*trpE5*) (9) to threonine independence with P1kc phage grown on strain HB94 (*hsdM*$^+$ *hsdR*$^-$ *thr*$^+$) as described by Wood (10). Strain KL380 (*alaS5 ara*$^-$ *argA*$^-$ *lacZ*$^-$ *leuB6 metE*$^-$ *recA*$^-$ *strA*$^-$) was obtained from K. B. Low; strain K-12 *hisB463* from K. Struhl and R. Davis; *E. coli* strains CV512 (*leuA371*/F$^+$), CV514 (*leuB401*/F$^+$), CV516 (*leuB61*/F$^+$), CV522 (*leuC222*/F$^+$), CV524 (*leuD211*/F$^+$), and *Salmonella typhimurium* strains *leuA124*, *leuB698*, *leuC5076*, and *leuD657* were obtained from J. Calvo (11, 12).

Media. LB broth, Vogel's, and Bonner's E media were used (13). Amino acids were added at 50 μg/ml. YPD medium contained 2% peptone, 1% yeast extract, and 2% glucose.

DNA Preparations. Covalently closed, supercoiled ColE1 DNA was prepared as described (6, 14). Yeast DNA was isolated from strain X2180-1A a (*SUC2 mal gal2 CUP1*) (obtained from the Yeast Genetics Stock Center, University of California, Berkeley). Yeast cells were grown in YPD medium to an OD_{600} of 7 and harvested by centrifugation. Twenty grams of cells was resuspended in 10 ml of buffer containing 0.5 M Na_4EDTA, 0.01 M Tris·HCl (pH 7), and 10 ml of proteinase K (2 mg/ml in the same buffer at pH 9.5), and 18 ml of sodium *N*-lauroylsarcosinate (1% in the same buffer, pH 9.5) was added. The cells were broken at 20,000 pounds/inch^2 (140 MPa) in a French pressure cell (American Instrument Co.). The lysate was then incubated at 50° for 12 hr. These buffers and the incubation procedure are described by Lauer and Klotz (5). The viscous lysate was extracted with redistilled phenol, and the DNA was precipitated by adding 2 volumes of ethanol and spooled out on a hooked glass rod. The DNA was further purified by isopropanol fractionation (15), digestion with pre-heated pancreatic RNase, phenol extraction, and a final isopropanol

fractionation. This DNA was used for the poly(dA)-tailing reaction.

DNAs from *E. coli* strain JA199 and its F^- derivative JA194 were prepared by standard procedures (14, 15). Cellular DNAs for reassociation experiments were banded in CsCl/ethidium bromide density gradients prior to use.

Preparation of Recombinant ColE1–Yeast DNAs and Transformation. Poly(dT)-tailed ColE1 DNA (L_{RI}) and poly(dA)-tailed sheared yeast DNA (average molecular weight = 8 to 10×10^6) were prepared as described by Clarke and Carbon (6, 14). Annealing of the two DNAs in an equimolar ratio produced a preparation with 10% circles, 16% tailed circles, 61% linears, and 14% tangles (visualized by standard electron microscopy).

Twenty-five micrograms of this annealed DNA preparation was used to transform strain JA199 to colicin E1 resistance as described by Clarke and Carbon (6), except that after the cells were diluted 10-fold with LB broth, they were grown for 90 min at 37° before plating in the presence of colicin E1.

Isolation of Recombinant Plasmid DNAs. A modification of the Hirt extraction procedure (16) was used. Cultures were incubated in the presence of chloramphenicol (200 μg/ml) to amplify plasmid for 16 hr, harvested, and treated with lysozyme/EDTA in the cold and lysed at room temperature with 1% sodium dodecyl sulfate for 15 min. The lysate was then kept at 0° for 30 min or until it was thoroughly chilled. The chromosomal DNA was pelleted by centrifugation of the lysate at 19,000 rpm in the Sorvall SS-34 rotor for 30 min. The supernatant liquid was slowly decanted, diluted 2-fold with STE buffer (10 mM Tris·HCl, pH 8, 10 mM NaCl, 1 mM EDTA), and made 1 M in NaCl. Phenol extraction was carried out for 20 min at 0°. The aqueous layer was removed, mixed with 2 volumes of cold absolute ethanol, and kept at −20° for 2 hr. The precipitate was collected by centrifugation, and purified by ethidium bromide/CsCl banding.

Labeling of Plasmid DNA and Reassociation Kinetics. DNA was labeled by nick translation with 30 μM deoxyribonucleoside triphosphates, [^{3}H]dTTP (8.4 Ci/mmol), and [^{3}H]dATP (8.3 Ci/mmol) by the method of M. Dieckmann and P. Berg (personal communication) as described by Schachat and Hogness (17). pY*eleu*10 DNA was labeled to a specific activity of 2.4×10^6 cpm/μg and pY*ehis*2 DNA to 2.2×10^6 cpm/μg.

The reassociation kinetics of plasmid DNAs at 65° in the presence and absence of various driver DNAs was measured by an S1 nuclease assay as previously described (17, 18).

Enzymes. *Eco*RI endonuclease and λ 5′-exonuclease were isolated as described (14); DNA polymerase I was a gift from A. Kornberg and deoxynucleotidyl terminal transferase was from R. Ratliff. S1 nuclease (200,000 units/mg) was purchased from Miles Laboratories, Inc.

Biohazard Considerations. Although F-mediated transfer of ColE1-like plasmids provides a valuable tool for the identification of complementing hybrid plasmids, the procedure converts normally non-transmissible plasmids into a state that permits ready transfer to other bacterial hosts. A recent revision (September, 1976) of the National Institutes of Health guidelines specifically prohibits the cloning of foreign DNA in K-12 hosts that contain wild-type conjugative plasmids. We have therefore discontinued the use of F-mediated transfer. Other procedures to detect complementation, such as isolation of hybrid plasmid DNA from F^- cells followed by transformation of suitable auxotrophic recipients, would appear to offer less risk of escape of the hybrids into the environment, and are in line with the current guidelines.

This work was carried out under P2 laboratory conditions. All bacteria and DNA preparations were destroyed by autoclaving or exposure to Clorox solution before disposal.

RESULTS

In order to set up a definitive test for the ability of any given eukaryotic gene system to be expressed and to complement an auxotrophic mutation in *E. coli*, the efficiency of the cloning procedure used must be high enough to insure that transformant clones containing hybrid DNA plasmids are obtained in numbers sufficient to be representative of the entire genome of the organism under study. In addition, it is preferable to use DNA segments produced by random scission (hydrodynamic shear), rather than by restriction endonuclease action, to ensure that the desired gene system remains intact on at least a portion of the cleaved DNA segments. For example, previous studies have shown that the use of the poly(dA-dT) "connector" method (7, 8) to join randomly sheared *E. coli* DNA with linear ColE1 DNA yields a preparation that will transform *E. coli* cells to colicin E1 resistance with high efficiency, thereby establishing a collection of transformants containing hybrid plasmids representative of the entire *E. coli* genome using only 10–15 μg of annealed DNA (6, 14).

A transformation of strain JA199 with 25 μg of poly(dT)-tailed plasmid ColE1 DNA (L_{RI}) annealed to poly(dA)-tailed sheared yeast DNA produced 190,000 colonies resistant to colicin E1. Since there was a doubling of cell numbers from the time of transformation to the plating of the transformed cells, this should represent 90,000 unique transformants. Plates from a control experiment in which cells were not exposed to DNA contained only a fifth as many colonies. Assuming that the haploid yeast genome contains 10^{10} daltons of DNA and that the sheared pieces of yeast DNA are 10^7 daltons in size, then only 4600 transformants would be necessary for a 99% probability that the pool of recombinant plasmids would contain any particular yeast DNA segment (6). Thus, the large pool of transformants ensures that we have cloned essentially all of the yeast genome and that a random selection of a few thousand transformants would be representative of the genome. Ten clones that were colicin-E1-resistant and E2-sensitive were picked at random and plasmid DNA was isolated. Eight of these yielded plasmid DNA that was larger than ColE1 DNA.

The colicin-resistant colonies were treated in three ways. A permanent bank of 4300 colonies was stored in 7% dimethylsulfoxide/LB broth cultures in microtiter dishes as described by Clarke and Carbon (6). Forty thousand colonies were also scraped off the plates and either plated out selecting for leucine-independent (Leu^+) cells on selective media, or used to isolate mixed plasmid DNA for subsequent transformations.

Hybrid ColE1–Yeast DNA Plasmids That Complement a *leuB* Mutation. The *leu-6* mutation present in *E. coli* strain JA199 and in all K-12 C600 strains is a lesion in the *leuB* gene, which encodes for β-isopropylmalate dehydrogenase (J. Calvo, personal communication; ref. 19). This enzyme catalyzes the oxidative decarboxylation of β-isopropylmalate to form α-keto-isocaproic acid, on the pathway of leucine biosynthesis in both *E. coli* and yeast.

Portions of a suspension of 40,000 JA199 transformant colonies containing hybrid ColE1–yeast DNA plasmids were plated on minimal medium with tryptophan and without leucine and incubated at 30°. After 3–4 days, large and small Leu^+ colonies appeared at a frequency of 10^{-6}. Several of the small colonies were selected and tested for resistance to colicins E1 and E2 (9). The plates were then further incubated at room

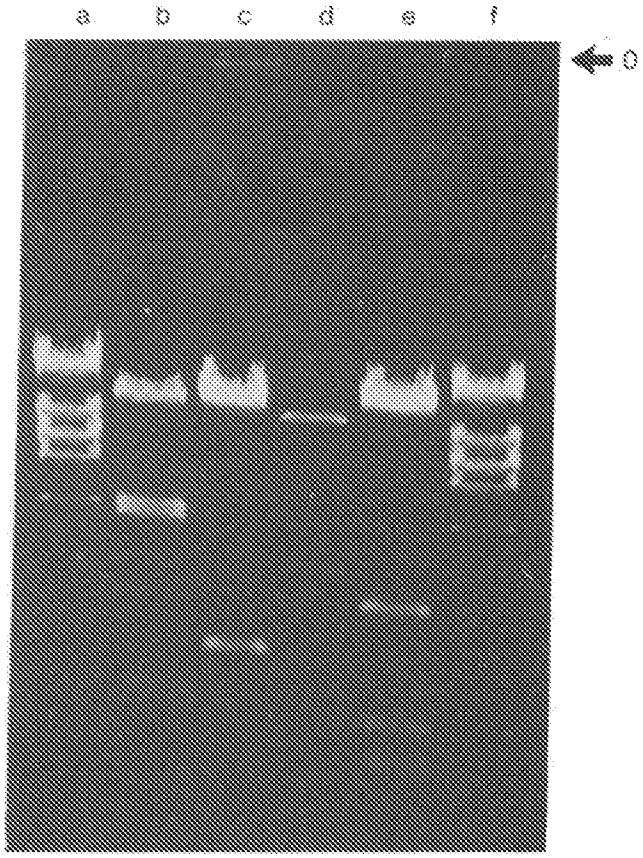

FIG. 1. Fractionation by 1.2% agarose gel electrophoresis of complete endonuclease *Eco*RI digests of various hybrid ColE1–yeast DNA plasmids; lanes a and f, phage λ DNA; lane b, pY*eleu*10 DNA; lane c, pY*eleu*11 DNA; lane d, pY*eleu*12 DNA; lane e, pY*eleu*17 DNA. Each well contained 1 μg of DNA digested with excess endonuclease *Eco*RI as previously described (1, 8). Electrophoresis was carried out at 8 V/cm for 2 hr and the DNA was visualized as described (1). The sizes of the λ fragments are (from the top, in daltons): 13.7×10^6, 4.7×10^6, 3.7 to 3.5×10^6, 3.0×10^6, and 2.1×10^6.

temperature for 2 weeks, after which time very small (1–2 mm) colonies appeared. Two of the intermediate-size Leu$^+$ colonies and 15 of the very small ones that were colicin-E1-resistant and E2-sensitive were also tested for the ability to donate their Leu$^+$ character to strain KL380 on minimal medium containing arginine, methionine, and streptomycin. All except one transferred the Leu$^+$ character at a high frequency (10^{-3}–10^{-4} per donor cell). The Leu$^+$ recipients were also colicin-E1-resistant and E2-sensitive, an indication that hybrid ColE1 plasmids had been transferred (9).

Covalently closed, supercoiled plasmid DNA was isolated from the 16 Leu$^+$ colicin-E1-resistant transformants described above. The purified plasmid DNAs were treated with excess endonuclease *Eco*RI and the digests were fractionated by electrophoresis on 1.2% agarose gels. Four different patterns of *Eco*RI restriction fragments were observed among this group (Fig. 1). Plasmid DNA (pY*eleu*10) from the fastest-growing Leu$^+$ transformant gave a unique fragment pattern, quite different from that displayed by any of the slower-growing transformants. The majority of the latter group gave the restriction pattern shown by pY*eleu*12 DNA, although two other plasmid types were obtained, as typified by pY*eleu*11 and pY*eleu*17 (Fig. 1). These four plasmids are of different sizes, with molecular weights ranging from 8.9×10^6 to 13.5×10^6 (see Table 1). None of the *Eco*RI fragments obtained from this plasmid group appear to be identical, although in each case the largest fragment is presumed to contain the 4.2×10^6 dalton ColE1 segment.

Table 1. Transformation of *E. coli* auxotrophs by ColE1–yeast hybrid plasmids

Plasmid DNA	Molecular weight $\times 10^{-6}$	Allele transformed	Transformants/μg DNA: Colicin-E1 resistant	Transformants/μg DNA: Leu$^+$ or His$^+$*
pY*eleu*10	13.5	*leuB6*	270,000	310,000
		leuB401	21,000	24,000
		leuB61	26,000	16,000
pY*eleu*11	8.9	*leuB6*	70,000	3,900
		leuB401	140,000	0
		leuB61	42,000	0
pY*eleu*12	10.3	*leuB6*	70,000	7,600
		leuB401	17,000	0
		leuB61	8,800	0
pY*eleu*17	13.2	*leuB6*	70,000	7,500
		leuB401	73,000	0
		leuB61	18,000	0
pY*ehis*1	10.7	*hisB463*	15,000	1,300*
pY*ehis*2	10.7	*hisB463*	26,000	1,700*
pY*ehis*3	10.7	*hisB463*	26,000	1,900*

These data have been corrected for the presence of both spontaneous Leu$^+$ revertants and colicin-E1-resistant mutants. Molecular weights were determined by electron microscopy using ColE1 DNA (4.2×10^6) as standard.
* Histidine-independent transformant.

These plasmid DNAs (pY*eleu*10, pY*eleu*11, pY*eleu*12, and pY*eleu*17) were used to transform three different *E. coli leuB* mutants, selecting for either Leu$^+$ or colicin E1 resistance. As shown in Table 1, plasmid pY*eleu*10 DNA is capable of transforming all of the *leuB* mutants (*leuB6*, *leuB61*, and *leuB401*) (11) to both Leu$^+$ and colicin E1 resistance with high frequency ($>10^5$ transformants per μg). Although the other three plasmid DNAs would readily transform all of the *leuB* strains to colicin E1 resistance, these plasmids would complement only the mutation in *leuB6* (Table 1). pY*eleu*10 DNA is not capable of transforming to Leu$^+$ strains bearing mutations in other genes of the *leu* operon, such as *leuA371*, *leuC222*, or *leuD211* (11).

If pY*eleu*10 carries a segment of yeast DNA that specifies the synthesis of a functional β-isopropylmalate dehydrogenase, it should complement deletions of the *leuB* region in the bacterial host cell. Although well-characterized *leuB* deletions in *E. coli* K-12 were not available, several *leu* deletion mutants in *Salmonella typhimurium* have been mapped by Calvo and Worden (12). The pY*eleu*10 plasmid was transferred from strain JA199 into the *Salmonella* deletion mutants *leuA124*, *leuB698*, *leuC5076*, and *leuD657* (obtained from Joseph Calvo), by F-mediated transfer (6). Leu$^+$ *Salmonella* colonies were obtained only from the JA199/pY*eleu*10 × *leuB698* cross, with a frequency of 10^{-3} Leu$^+$ recipients per donor cell. Plasmid DNA isolated from four of the *Salmonella* Leu$^+$ strains gave an *Eco*RI restriction fragment pattern identical to that from authentic pY*eleu*10 DNA. Thus, the presence of pY*eleu*10 DNA correlates with the transfer of a Leu$^+$ phenotype to a strain harboring a deletion in *leuB*.

Isolation of Hybrid Plasmids That Complement a *hisB* Deletion. Mixed plasmid DNA isolated from the 40,000

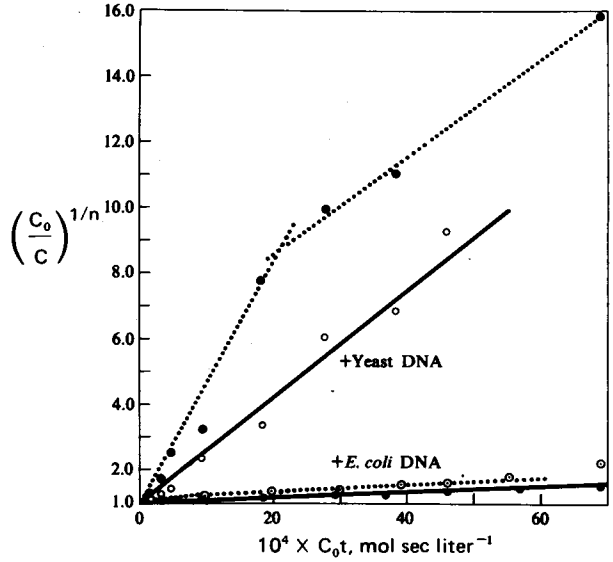

FIG. 2. Reassociation kinetics of labeled single-stranded pYe*leu*10 DNA fragments (0.085 μg/ml) in the presence of single-stranded fragments of: (●——●) salmon sperm DNA, 1000 μg/ml; (⊙···⊙) *E. coli* DNA, 196 μg/ml; (○——○) yeast (*S. cerevisiae*) DNA, 176 μg/ml; and (⊕···⊕) yeast DNA, 526 μg/ml. The experiment was carried out as described in *Methods*. C, DNA concentration in moles of nucleotide per liter; C_0, initial DNA concentration; t, time in seconds; *n*, 0.45 (18).

transformant colonies was used in attempts to transform several *E. coli* auxotrophic strains to prototrophy. Among these strains was *hisB463*, which lacks an active imidazole glycerol phosphate dehydratase. Struhl *et al.* (4) have previously isolated a λ phage containing a segment of yeast DNA (λgt-Sc*his*) that suppresses this mutation. Two micrograms of our mixed hybrid plasmid DNA was used to transform strain *hisB463*, selecting for His$^+$. Three His$^+$ colonies were detected after 5 days of incubation at 30°, as compared to 10^5 colicin-E1-resistant colonies. These colonies were colicin-E1-resistant, but sensitive to colicin E2. Plasmid DNAs were isolated from these strains and used to transform strain *hisB463* again. As shown in Table 1, these purified plasmid DNAs (pYe*his*1, pYe*his*2, and pYe*his*3) transformed the *hisB463* strain to colicin E1 resistance and to His$^+$ with high frequency. Transformation to His$^+$ occurred at a lower frequency than to colicin E1 resistance, although prolonged incubation produced more His$^+$ colonies.

All three pYe*his* plasmids are of similar size, with a molecular weight of 10.7×10^6. They each contain a single *Eco*RI endonuclease restriction site within the segment of cloned yeast DNA, and are probably identical.

The pYe*leu*10 and pYe*his*2 Plasmids Contain Yeast DNA. We have shown that purified pYe*leu*10 and pYe*his* DNAs can suppress the appropriate *leu* or *his* mutation in *E. coli*; it is also necessary to establish the source of the cloned DNA segments. In order to prove that these plasmids contain segments of yeast DNA, we labeled pYe*leu*10 and pYe*his*2 DNAs by nick translation with DNA polymerase I and ^{3}H-labeled deoxynucleoside triphosphates and determined if single-stranded plasmid DNA would reassociate with authentic yeast DNA.

The reassociation of single-stranded pYe*leu*10 DNA was driven well by yeast DNA single strands, but not appreciably by single-stranded *E. coli* DNA (Fig. 2). If one assumes that 70% of pYe*leu*10 is yeast DNA (from the total pYe*leu*10 molecular weight, 13.5×10^6, minus the ColE1 segment, 4.2×10^6), then we can calculate from the initial slopes of the reassociation rate curves that the yeast segment in pYe*leu*10 is 0.11–0.12% of the

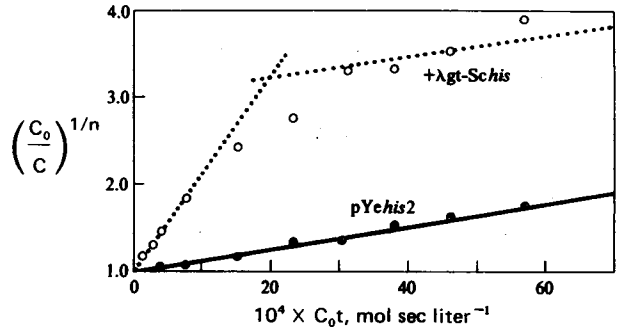

FIG. 3. Reassociation kinetics of labeled single-stranded pYe*his*2 DNA fragments (0.07 μg/ml) in the presence of single-stranded fragments of: (⊕——⊕) salmon sperm DNA, 1000 μg/ml; and (○···○) λgt-Sc*his* DNA, 1.25 μg/ml. See *Methods* for details.

yeast genome. If we further assume one copy of the pYe*leu*10 segment in the yeast genome, then the total yeast genome would be 7.8 to 8.5×10^9 daltons, well within the range of values in the literature (5 to 10×10^9 daltons) (5). Apparently this segment of DNA is a unique fragment of the yeast genome.

Fig. 3 shows the reassociation kinetics of single-stranded pYe*his*2 DNA in the presence or absence of single-stranded λgt-Sc*his* DNA, from the recombinant λ phage containing a segment of yeast DNA that suppresses the *hisB463* mutation (4). If the pYe*his*2 plasmid and λgt-Sc*his* contain similar segments of yeast DNA, then the addition of λgt-Sc*his* single-stranded DNA should increase the rate of reassociation of single-stranded pYe*his*2 DNA. As shown in Fig. 3, λgt-Sc*his* DNA markedly increases the rate of reassociation of pYe*his*2 DNA. The fraction of λgt-Sc*his* DNA that is homologous to the pYe*his*2 DNA can be calculated as above to be equal to 22% of the λgt-Sc*his* DNA (30×10^6 daltons), equivalent to 6.6×10^6 daltons of DNA. From the molecular weights of pYe*his*2 and ColE1 DNAs and assuming a full-length ColE1 segment is present in pYe*his*2, the calculated mass of the cloned yeast DNA segment would be 6.7×10^6 daltons. pYe*his*2 DNA contains a single *Eco*RI site, which is apparently identical to one of the two sites defining the yeast segment in λgt-Sc*his* DNA.

Efficiency of the Complementation. One measure of the efficiency of complementation or suppression of an *E. coli* mutation by a cloned yeast DNA segment would be the relative growth rates of the transformant and wild-type cells in minimal unsupplemented media. For example, *hisB463* strains containing the λgt-Sc*his* prophage are reported to grow with a 2.7 hr generation time in minimal medium without histidine, versus a 2.1 hr generation time for a *his*$^+$ wild-type under the same conditions (4). Colonies of *leuB*/pYe*leu*10 strains growing on minimal solid media without leucine appear to grow at about 1/3–1/2 the rate of wild-type colonies. In liquid minimal media without leucine, a *leuB*$^+$ transductant of a *leuB6* strain grew twice as fast (1 hr doubling time) as did strain *leuB6*/pYe*leu*10 (2 hr doubling time). However, in media supplemented with leucine, both strains grow with a 1 hr doubling time. Apparently, the presence of the yeast DNA segment in pYe*leu*10 partially compensates for the *leuB* mutation, but has no deleterious effects on the bacterial cell.

leuB6 strains bearing the pYe*leu*11, pYe*leu*12, and pYe*leu*17 plasmids grow very slowly when plated on solid media without leucine, requiring 3–4 days at 30° to form tiny colonies just visible to the naked eye. Apparently the suppression effect associated with the presence of these plasmids in *leuB6* strains is quite inefficient.

Although the selections for the pYe*leu* and pYe*his* containing

strains were carried out at 30° (the optimum temperature for yeast growth), strains bearing these plasmids grew equally well at 37° on selective media.

DISCUSSION

It now seems clear that segments of yeast DNA capable of complementing a variety of *E. coli* mutations can be readily isolated. The actual mechanism of complementation by the cloned DNA is not clear. The *hisB463* mutation that is complemented by the yeast DNA cloned in pYe*his*2 and λgt-Sc*his* is thought to be a deletion (4). The *leuB6* mutation in strain JA199 is a point mutation, but we have observed high frequency transfer of the Leu$^+$ phenotype to a *Salmonella typhimurium leuB* deletion by F-mediated transfer from a pYe*leu*10 strain. However, even if a deletion mutation is complemented, we are not certain that the yeast DNA in pYe*leu*10 contains the homologous gene from yeast, *leu2* (20). The different milieu of the *E. coli* cell may allow a product from some other yeast gene to function as a β-isopropylmalate dehydrogenase.

Certainly the mechanism of this type of complementation or suppression can differ in various cases. For example, in addition to pYe*leu*10, three other hybrid ColE1–yeast DNA plasmids have been found that suppress the *leuB6* mutation, although with a much lower efficiency than does pYe*leu*10. These plasmids all display different patterns of cutting by endonuclease *Eco*RI. Although pYe*leu*10 will strongly complement any of the *leuB* mutations we have tried (Table 1), these other pYe*leu* plasmids appear to suppress only the *leuB6* mutation in C600 strains. The possibility exists of missense suppression mediated by yeast tRNA or aminoacyl-tRNA synthetases produced from yeast genes on the plasmid, since it is well known that tRNA misaminoacylation occurs readily in *E. coli*–yeast heterologous systems (21).

We believe, then, that many yeast DNA segments in *E. coli* will be readily expressed; the barriers to translation and transcription are somehow overcome. However, not all genes will be expressed similarly. For example, the pYe*his*2 plasmid and λgt-Sc*his* phage are expressed somewhat more efficiently than is pYe*leu*10, as measured by growth rates of transformants on unsupplemented minimal media. pYe*his*2 transformants to His$^+$ do not occur at the same frequency as do transformants to colicin E1 resistance; few His$^+$ transformants appear at first but increasing numbers appear with longer incubation times. pYe*leu*10 transformants to Leu$^+$ and to colicin E1 resistance do occur at the same frequency, and they appear more synchronously. These differences could be due to differing rates of expression as alluded to by Struhl *et al.* (4) or to the expression of other genes on the plasmids that hinder cell growth in the case of pYe*his*2.

The level of success with which we have found hybrid ColE1–yeast DNA plasmids that suppress *E. coli* mutations is fairly good. Out of 11 different mutations tried with our recombinant collection, two were successfully suppressed and another two to three show apparent complementation, but have not yet been completely characterized. In one case, a *trpAB* deletion has been shown to be suppressed by a ColE1–yeast recombinant plasmid, and the cloned DNA segment has been shown to be yeast DNA. Thus, it seems possible that productive expression of yeast (and perhaps other eukaryotic) DNA in *E. coli* can occur more readily than has previously been anticipated.

We are grateful to Denise Richardson and Gary Tschumper for valuable technical assistance; to K. Struhl and R. Davis for a gift of λgt-Sc*his* DNA, and to J. Calvo for supplying several key *leu* mutant strains. Strain JA199 was constructed by Margaret Nesbitt. This work was supported by Research Grants CA-11034 and CA-15941 from the National Cancer Institute, U.S. Public Health Service, and by a research grant from Abbott Laboratories.

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TAB I

Proc. Natl. Acad. Sci. USA
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Genetics

Expression in *Escherichia coli* K-12 of the structural gene for catabolic dehydroquinase of *Neurospora crassa*

(recombinant DNA/pBR322 plasmid/eukaryotic gene regulation/*qa* cluster)

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Contributed by Norman H. Giles, June 6, 1977

ABSTRACT **The inducible quinic acid catabolic pathway of *Neurospora crassa* is controlled by four genes, the *qa* cluster which includes structural genes *qa-2, qa-3, qa-4* for three enzymes and a regulatory gene, *qa-1*. In this paper we report the molecular cloning of at least the *qa-2* gene which encodes the catabolic dehydroquinase (5-dehydroquinate hydro-lyase, EC 4.2.1.10). Endo·R·*Hin*dIII restriction endonuclease fragments of *N. crassa* DNA from a *qa-1*^{C} (constitutive) mutant and of *Escherichia coli* plasmid pBR322 DNA were ligated *in vitro* and used to transform an *aroD6* (5-dehydroquinate hydrolyase deficient) strain of *E. coli* K12. The recombinant plasmid (pVK55) isolated from one AroD^{+} transformant (SK1518) contained, in addition to pBR322, two *N. crassa Hin*dIII fragments with molecular weights of 2.3×10^{6} and 1.9×10^{6}. Derivatives of SK1518 cured of plasmid DNA were phenotypically Amp^{S} and AroD^{-}. These cured strains, retransformed with pVK55, were phenotypically Amp^{R} and AroD^{+}. Strains transformed with pVK55 possessed 5-dehydroquinate hydrolyase activity but no activity was present in any AroD^{-} strain. The enzyme extracted from strains containing the recombinant plasmid was identical to *N. crassa* catabolic dehydroquinase by the criteria of heat stability, ammonium sulfate fractionation, immunological crossreactivity, molecular weight, and purification characteristics. This identity demonstrates that the *N. crassa qa-2*$^{+}$ gene is carried by the recombinant plasmid and is apparently transcribed and translated with complete fidelity. Furthermore, subunit assembly of the *N. crassa* polypeptides also occurs in *E. coli*, because the catabolic dehydroquinase is a multimer composed of approximately 20 identical subunits.**

Recent progress in the elucidation of molecular mechanisms involved in genetic regulation in prokaryotes has depended on the isolation of DNA sequences carrying both structural and regulatory genes. Purification of these DNA sequences has led to the establishment of *in vitro* systems in which the mode of action of regulatory proteins could be directly tested (1) and, in addition, has permitted the sequencing of promotor and operator regions (2–4). By using molecular cloning techniques, it should now be possible to isolate similar DNA sequences from eukaryotes. Of particular significance is the recent demonstration by Struhl *et al.* (5) and Ratzkin and Carbon (6) that either λ or plasmid hybrids containing specific yeast genes can be selected by their ability to complement auxotrophic mutants of *Escherichia coli*. Cloned DNA sequences obtained by this method will be useful for the study of eukaryotic gene regulation only if they contain both structural and regulatory genes.

One such DNA sequence is the *qa* gene cluster of *Neurospora crassa* which controls the first three reactions in the inducible quinic acid catabolic pathway (Fig. 1A) (7, 8). Three of the loci are the structural genes for the individual *qa* enzymes: *qa-2*, catabolic dehydroquinase (5-dehydroquinate hydro-lyase, EC 4.2.1.10); *qa-3*, quinate dehydrogenase (quinate:NAD^{+} oxidoreductase, EC 1.1.1.24); and *qa-4*, dehydroshikimate dehydrase. The fourth gene, *qa-1*, encodes a regulatory protein which in conjunction with the inducer, quinic acid, controls the expression of the three structural genes. The catabolic dehydroquinase enzyme of *N. crassa* has been well characterized (9) and is capable of complementing aromatic amino acid auxotrophs deficient in the biosynthetic dehydroquinase isozyme (10). This combination of structural genes under the control of a tightly linked regulatory locus and a well-defined enzymatic activity (catabolic dehydroquinase) which should complement an *aroD*$^{-}$ mutant of *E. coli* (see Fig. 1) makes the *qa* gene cluster an ideal DNA sequence for cloning on a recombinant plasmid.

In this communication we describe the isolation and preliminary characterization of a pBR322–*N. crassa* DNA recombinant plasmid that complements an *aroD6* (5-dehydroquinate hydrolyase deficient) strain of *E. coli*. The transformed strains contain a 5-dehydroquinate hydrolyase activity which has been shown to be biochemically and immunologically identical to the *N. crassa* catabolic dehydroquinase.

MATERIALS AND METHODS

Materials. Reagents were obtained from the following sources: CsCl (high purity), Penn Rare Metals; Sarkosyl NL-97, Geigy Chemical Co.; lysozyme, Worthington Biochemical Corp.; "ultra pure" $(NH_4)_2SO_4$ and enzyme grade sucrose, Schwarz/Mann; agarose, Seakem Laboratories; ethidium bromide, Calbiochem; bovine serum albumin, Miles Research Laboratories; goat anti-rabbit antiserum, Grand Island Biological Co.; chloramphenicol, tetracycline, streptomycin sulfate, and RNase IIA, Sigma Chemical Co.; ampicillin (Omnipen), Wyeth Pharmaceutical Co. Spectinomycin sulfate was the generous gift of the Upjohn Co. T4 DNA ligase was purified by the method of Weiss *et al.* (11). Endo·R·*Hin*dIII was obtained from New England Biolabs, Inc. Endo·R·*Eco*RI was purified by the method of Green *et al.* (12). One unit of either enzyme is defined as that amount required to completely digest 1 μg of λ DNA in 1 hr at 37°. All other chemicals were reagent grade. Bacteriophage λ DNA was graciously supplied by R. Mural.

Bacterial Strains and Media. The relevant genotypes and origins of the bacterial strains are listed in Table 1. Bacterial nomenclature conforms to the suggestions of Demerec *et al.* (13) and Bachmann *et al.* (14).

The procedure and media for conjugational crosses were those of Willetts *et al.* (15). Specific genotypic characteristics were determined by using the replica plating techniques of Clark and Margulies (16). The presence or absence of the *hsdR4*

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Table 1. Bacterial strains

Strain	Sex	*proA*	*argE*	*his*	*aroD*	*rpsE*	*rpsL*	*hsdR*	*hsdM*	Other	Source*
AB1360	F⁻	2	3	4	6	+	+	+	+		B. Bachmann
C600	F⁻	+	+	+	+	+	+	+	+	*thr, leu*, pBR322	D. Vapnek
HB94	Hfr	+	+	+	+	+	+	4	+		B. Bachmann
KL208	Hfr	+	+	+	+	+	+	+	+	Origin, 30 min	K. B. Low
KL226	Hfr	+	+	+	+	+	+	+	+	Origin, 15 min	K. B. Low
SK274	F⁻	2	3	4	6	−	+	+	+		A
SK1478	F⁻	+	3	4	6	−	+	4	+		B
SK1485†	F⁻	+	3	4	6	−	−	4	+		C
SK1516†	F⁻	+	3	4	6	−	−	4	+	pVK53	This paper
SK1518	F⁻	+	3	4	6	−	−	4	+	pVK55	This paper
SK1520	F⁻	+	3	4	6	−	−	4	+		This paper
SK1524†	F⁻	+	3	4	6	−	−	4	+	pVK55	This paper
SK1529	F⁻	+	3	4	6	−	−	4	+	pVK55	This paper

* A = Spc^R mutant of AB1360 obtained with ethyl methanesulfonate; B = SK274 × HB94 Pro$^+$ conjugant; C = Str^R mutant of SK1478 obtained with ethyl methanesulfonate.

† These strains contain an unknown mutation that prevents high levels of complementation of the *aroD6* allele in the presence of pVK55. This mutation has reverted or been suppressed in SK1518 and its derivatives.

allele was tested for by the ability to plate Plvir·K or Plvir·B. Cells were grown in Luria (L) broth (17) or K medium (18). Minimal medium consisted of M56/2 buffer (15) supplemented with glucose (0.5%), appropriate amino acids (50 μg/ml), thiamin (0.001%), and ampicillin (20 μg/ml). AroD$^+$ transformants were selected by the ability of colonies to grow on minimal agar plates lacking aromatic amino acids.

Purification of Plasmid and *N. crassa* DNA. Plasmid pBR322 DNA was obtained from *E. coli* C600 (pBR322) grown in L broth (plus ampicillin, 20 μg/ml) by the method of Vapnek *et al.* (19). *N. crassa* DNA was isolated from a *qa-1^C* (constitutive) mutant, strain M105-R12-1.5 (20), by the method of Hautala *et al.* (21). The DNA was further purified by digestion with preheated RNase followed by Sepharose 4B chromatography in 10 mM Tris·HCl, pH 7.5/1 mM EDTA/0.2% Sarkosyl.

Construction of Recombinant pBR322 Plasmids. *N. crassa* DNA and pBR322 DNA were simultaneously digested to completion with Endo·R·*Hin*dIII. The reaction mixture (340 μl) containing 32 μg of *N. crassa* DNA, 10 μg of pBR322 DNA, 6 mM Tris·HCl, (pH 7.5), 50 mM NaCl, 6 mM $MgCl_2$, 40 μg of bovine serum albumin, and 100 units of Endo·R·*Hin*dIII was incubated for 75 min at 37°. After heating at 65° for 10 min to inactivate the enzyme, the mixture was further incubated for 5 min at 37° prior to addition of phage T4 ligase. Ligation was performed in a final volume of 400 μl by the method of Tanaka and Weisblum (22), followed by dialysis for 4 hr against 1 liter of 10 mM Tris·HCl, pH 7.0/1 mM EDTA.

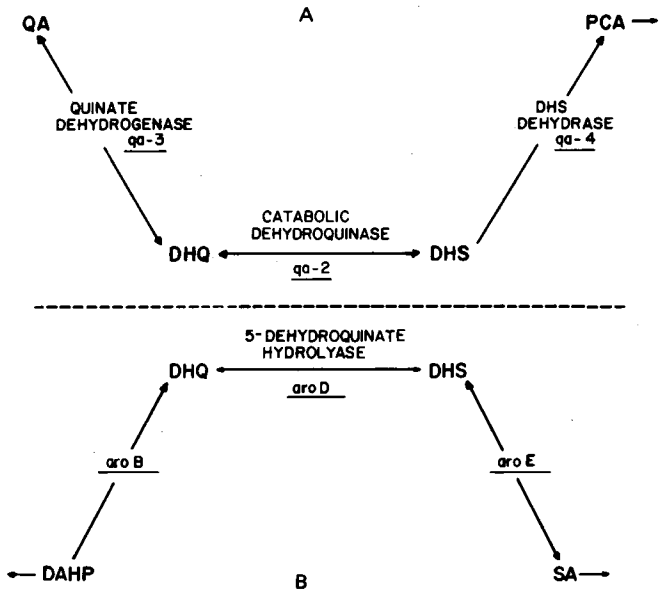

FIG. 1. Relationship of the initial reactions of the inducible quinic acid catabolic pathway in *N. crassa* (*A*) with the initial reactions of the aromatic amino acid biosynthetic pathway in *E. coli* (*B*). Abbreviations: QA, quinic acid; PCA, protocatechuic acid; DHQ, dehydroquinic acid; DHS, dehydroshikimic acid; DAHP, 3-deoxy-D-arabinoheptolusonic acid 7-phosphate; SA, shikimic acid.

Bacterial Transformation. Recipient strains were transformed as described (19) except that DNA uptake was allowed to take place during a 1-min incubation at 43.5°. After growth at 37° in L broth for 18 hr, the cells were concentrated by centrifugation such that approximately 2 × 10^8 cells were plated on each minimal agar selective plate (plus ampicillin, 20 μg/ml). Some cells were plated on L agar plates containing ampicillin (20 μg/ml). The transformation proficiency of the initial experiment was approximately 1 × 10^4 transformants per μg of DNA.

Transformation of recipient strains with purified recombinant plasmid DNAs was carried out by the same technique except that 0.5 μg of DNA was used per 10^8 cells and overnight expression was omitted. In addition, the cells were washed twice with M56/2 buffer prior to plating on minimal selective agar plates.

Purification and Analysis of Recombinant Plasmid DNAs. Recombinant plasmid DNAs were purified as described by Vapnek *et al.* (19). Plasmid DNAs were digested with Endo·R·*Hin*dIII and electrophoresed on 0.8% agarose gels as outlined (19).

Biochemical Procedures. The assay procedure for 5-dehydroquinate hydrolyase has been reported (23). One unit of activity represents 1 nmol of dehydroshikimate produced per min at 37°.

For enzyme assays, 500-ml cultures were grown in L broth (plus ampicillin, 20 μg/ml) to 2.5 × 10^8 cells per ml, washed with 50 mM Tris·HCl, pH 7.5/10% sucrose, and stored at −70°. The cells were lysed according to the procedure of Wickner *et al.* (24) with the following modifications. The frozen cell pellet was resuspended in 50 mM Tris·HCl, pH 7.5/10% sucrose (0.75 g wet weight of cells per 10 ml of buffer), frozen in a −50° dry ice/ethanol bath, and thawed at 20°. To each 10 ml of cell suspension were added 1.25 ml of 0.1 M EDTA, 0.25 ml of 4 M

NaCl, and 1.0 ml of lysozyme (2.5 mg/ml in 0.25 M Tris·HCl, pH 7.5). After 30 min at 0° and 20 min at 37°, the suspension was centrifuged at 100,000 × *g* for 60 min, and the clear supernatant was made 0.4 mM in dithiotreitol and 0.1 mM in phenylmethylsulfonyl fluoride.

Thermal stability studies were performed by heating the crude supernatants at 71° for 10 min. The 0–50% ammonium sulfate fractions were obtained from the crude, unheated supernatants. The precipitates were dissolved in buffer A (10 mM potassium phosphate, pH 7.5/0.4 mM dithiothreitol/0.1 mM EDTA/0.1mM phenylmethylsulfonyl fluoride and dialyzed overnight against the same buffer prior to assay. Double-immunoprecipitation experiments were performed using the dialyzed ammonium sulfate fractions or, in the case of SK1313, the crude supernatant was dialyzed against buffer A. The samples were treated with the gamma globulin fraction of rabbit antiserum prepared against pure catabolic dehydroquinase from *N. crassa* (25) followed by treatment with goat anti-rabbit antiserum (26).

The 5-dehydroquinate hydrolyase activity from the transformed strain SK1518 was purified according to the procedure reported (9) for the isolation of catabolic dehydroquinase from *N. crassa* (9) except that the RNase/DNase treatment was omitted and the activity was precipitated with 50% ammonium sulfate.

Protein concentrations were determined by the microbiuret (27) and Lowry (28) techniques. Analytical polyacrylamide disc gel electrophoresis was performed according to the method of Davis (29). Sucrose density gradient centrifugations were performed according to the method of Martin and Ames (30) at 37,000 rpm and 4° for 18 hr in an SW 65 rotor.

Containment. The experiments were carried out under P2/EK1 conditions as specified by the "National Institutes of Health Guidelines for Recombinant DNA Research."

RESULTS

Construction of Hybrid Plasmids and Bacterial Transformation. pBR322 is a ColE1 related plasmid (molecular weight 2.6×10^6) that carries resistance to ampicillin (Amp^R) and tetracycline (Tc^R) and contains a single Endo·R·*Hin*dIII cleavage site (31). Because Endo·R·*Hin*dIII cleaves within the promotor for the Tc^R gene, introduction of foreign DNA into this site results in sensitivity to tetracycline and facilitates the identification of hybrid plasmids (31).

The mixture of *N. crassa* nuclear DNA and plasmid pBR322 DNA prepared as described in *Materials and Methods* was used to transform an *aroD6* (5-dehydroquinate hydrolyase deficient), *hsdR4* (restriction deficient) strain of *E. coli* K12 (SK1485). Apparent complementation of the *aroD6* allele was detected by plating transformed cells on minimal agar plates lacking aromatic amino acids. Presumptive transformants that appeared were tested for the other chromosomal markers carried by SK1485 as well as for the phenotypic properties expected of strains carrying a pBR322 recombinant plasmid (Amp^R, Tc^S).

Approximately 2×10^4 colonies appeared, and 2000 were tested. Of these, one had the properties expected of a strain carrying a pBR322 recombinant plasmid that could complement the chromosomal *aroD6* mutation. Although this isolate initially grew slowly on minimal agar medium, upon purification one faster growing colony appeared. This colony (SK1518) was further purified for analysis.

Electrophoretic Analysis of Recombinant Plasmid DNA. Plasmid DNA was prepared from SK1518 and from a strain carrying a putative hybrid plasmid that did not complement the *aroD6* mutation (SK1516). The purified DNAs, along with pBR322 DNA, were analyzed by agarose slab gel electrophoresis after Endo·R·*Hin*dIII digestion. λ DNA treated with Endo·R·*Eco*RI was included as a molecular weight standard (Fig. 2, lane A). Treatment of pBR322 with Endo·R·*Hin*dIII yielded a single DNA band of 2.6×10^6 daltons (Fig. 2, lane B). When the plasmid obtained from SK1518 (pVK55) was digested with Endo·R·*Hin*dIII, three distinct fragments appeared (Fig. 2, lane D). One of these corresponded to pBR322, while the other two were calculated to be 1.9×10^6 and 2.3×10^6 daltons, based on the λ molecular weight standard. Cleavage of the plasmid from SK1516 (pVK53) with Endo·R·*Hin*dIII produced two DNA bands, one corresponding to pBR322 and a small DNA fragment of about 3×10^5 daltons (Fig. 2, lane E).

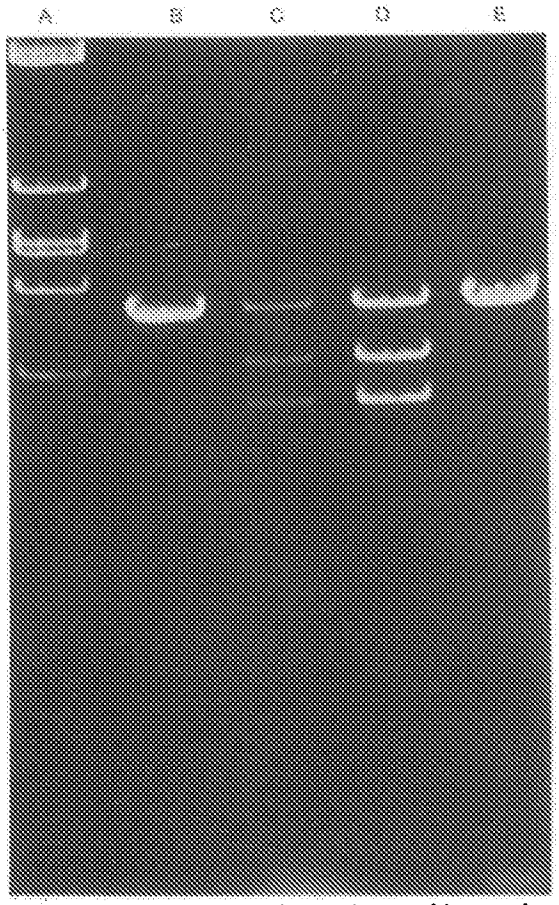

FIG. 2. Electrophoretic analysis of recombinant plasmids. Plasmid DNAs were isolated and digested with Endo·R·*Hin*dIII (B-E) as described in *Materials and Methods*. Lanes: A, λ DNA digested with Endo·R·*Eco*R1; B, pBR322; C, pVK55 from SK1524; D, pVK55 from SK1518; E, pVK53.

Properties of pVK55. To confirm that pVK55 carried the gene that resulted in complementation of the *E. coli aroD6* allele, SK1518 was cured of the plasmid by overnight growth at 44° in the presence of acridine orange (50 μg/ml). The cells were subsequently plated on L agar plates and tested for the appropriate phenotypic properties. Of 81 surviving colonies tested, 4 were sensitive to ampicillin and unable to grow in the absence of aromatic amino acids.

One of these cured derivatives (SK1520) and the original *aroD6* strain (SK1485) were used as recipients for transformation with pVK55 plasmid DNA. All Amp^R transformants of SK1485 analyzed ($n = 200$) grew extremely poorly in the absence of added aromatic amino acids (750-min doubling time), whereas the Amp^R transformants of SK1520 grew as well

Table 2. Levels of 5-dehydroquinate hydrolyase activity from *N. crassa* and various *E. coli* derivatives

Strain	Genotype	Plasmid	Total activity,* units	Total protein, mg	Specific activity, units/mg
SK1313	*aroD*+	—	1.86	44.2	0.042
SK1516	*aroD6*	pVK53	<0.03	44.7	<0.0007
SK1518	*aroD6*	pVK55	0.44	35.0	0.013
SK1520	*aroD6*	—	<0.03	52.0	<0.0006
SK1524	*aroD6*	pVK55	0.42	53.1	0.008
SK1529	*aroD6*	pVK55	0.48	47.9	0.010
M16†	*qa-2*+	—	2.4	76.5	0.031

* Determined as described in *Materials and Methods.*
† Strain M16 of *N. crassa* (32), induced with quinic acid.

Table 3. Comparison of 5-dehydroquinate hydrolyase activity from *N. crassa* and various *E. coli* strains

Strain	Plasmid	% recovery of activity: Heat shock	Ammonium sulfate* Pellet	Ammonium sulfate* Supernatant	Immunoprecipitate
SK1313	—	1.5	1.6	86	0
SK1518	pVK55	80	46	4	96
SK1524	pVK55	87	60	9	96
SK1529	pVK55	100	58	0	94
M16†	—	99	78	3	94

* 50% saturation; see *Materials and Methods.*
† Strain M16 of *N. crassa* (32), induced with quinic acid.

on minimal medium as SK1518 (136-min doubling time). In contrast, an *aroD*+ strain (SK1313) had a doubling time of 66 min, whereas the *aroD6* mutant (SK1485) showed no detectable growth without the addition of aromatic amino acids.

When the plasmids isolated from retransformants of SK1485 (SK1524) and SK1520 (SK1529) were treated with Endo·R·*Hin*dIII and examined by gel electrophoresis, they were found to be identical to pVK55. The electrophoretic pattern of one of these, the plasmid from SK1524, is shown in Fig. 2, lane C.

These results suggested that a plasmid-carried activity was responsible for the complementation of the *aroD6* allele but that the extent of complementation depended on the presence or absence of an additional chromosomal mutation. In order to confirm this hypothesis, both SK1518 and SK1524 were mated with either KL208 or KL226 donor Hfr strains. Among the Arg^+, Str^R recombinants obtained in a cross of SK1524 and KL208, greater than 90% showed high levels of complementation, whereas all the conjugants obtained with KL226 retained their poor growth characteristics in the absence of aromatic amino acids. No alteration in the level of complementation was observed when SK1518 was used as a recipient in similar crosses. These results suggested that SK1485 carried a second mutation that prevented a high level of complementation of the *aroD6* mutation by the pVK55 recombinant plasmid.

Characterization of Dehydroquinate Hydrolyase Activity. Several *E. coli* strains transformed with either pVK53 or pVK55 were lysed and assayed for 5-dehydroquinate hydrolyase activity. The *AroD*− strains tested [SK1516 (pVK53) and SK1520] showed no detectable 5-dehydroquinate hydrolyase activity (Table 2). In contrast, three derivatives carrying pVK55 (SK1518, SK1524, and SK1529) all contained significant levels of enzyme although the activities were lower than those from an *aroD*+ *E. coli* control strain (SD1313) or a quinic acid induced strain (M16) of *N. crassa*.

The characteristics of the 5-dehydroquinate hydrolyase activity obtained from the *AroD*+ transformants (SK1518, SK1524, and SK1529) were compared with those of the wild-type enzyme from *E. coli* and the catabolic enzyme from *N. crassa* (Table 3). By the criteria of heat stability, ammonium sulfate fractionation, and immunoprecipitation, the enzyme activity isolated from pVK55 transformed strains of *E. coli* appeared identical to *N. crassa* catabolic dehydroquinase. The biosynthetic 5-dehydroquinate hydrolyase obtained from SK1313 (*aroD*+) was significantly different by each of these criteria. An additional distinction between the two enzymes is the native molecular weight. The *E. coli* biosynthetic enzyme has a reported molecular weight of approximately 40,000 (33), whereas the catabolic enzyme from *N. crassa* is considerably larger, 220,000 (9). Sucrose density gradient centrifugation demonstrated that the enzyme obtained from pVK55 transformed strains had a molecular weight identical to the *N. crassa* catabolic dehydroquinase (Fig. 3).

5-Dehydroquinate hydrolyase was further purified from SK1518 by the procedure described for the *N. crassa* catabolic enzyme (9). The activity obtained behaved identically to the catabolic *N. crassa* enzyme with respect to both Sephadex G-200 gel filtration and DEAE-cellulose chromatography. The active fractions obtained from the DEAE-cellulose column were pooled, concentrated, and analyzed by polyacrylamide gel electrophoresis. A single protein species, which comigrated with *N. crassa* catabolic dehydroquinase, was observed.

DISCUSSION

The results presented in this communication demonstrate unequivocally that eukaryotic DNA can be faithfully transcribed and translated in a prokaryotic host. The presence of functional *N. crassa* catabolic dehydroquinase in *E. coli* also demonstrates that assembly of subunits takes place, because the *N. crassa* enzyme is a multimer composed of approximately 20 10,000-dalton subunits (9).

The *N. crassa* DNA cloned in pVK55 was isolated from a strain carrying a *qa-1*^{C} mutant (constitutive for synthesis of catabolic dehydroquinase). Because the *qa* cluster is under positive control exerted by the *qa-1* gene, transcription and translation of the *qa-2*+ gene in this mutant occurs only if the *qa-1*^{C} gene is expressed. However, when carried by the recombinant plasmid, the *qa-2*+ gene could be expressed by one of three alternative mechanisms: (*i*) transcription initiation from a pBR322 promoter; (*ii*) transcription initiation from a normal *N. crassa* promoter by a mechanism that does not require the presence of a regulatory protein: (*iii*) transcription initiation by the normal *N. crassa* mechanism which requires the presence of an expressed *qa-1*^{C} gene. If the third mechanism should prove to be the one operating, pVK55 must contain the entire *qa* cluster because *qa-1* and *qa-2* are the proximal and distal genes of the cluster (34). Although there may be enough *N. crassa* DNA contained in pVK55 to encode for the entire *qa* cluster, initial experiments have not demonstrated the presence of the *qa-4* gene (dehydroshikimate dehydrase) which is adjacent to *qa-2* (34). Furthermore, it is not clear if the two Endo·R·*Hin*dIII fragments carried by pVK55 are in fact contiguous on the *N. crassa* chromosome. Preliminary experiments suggest that a plasmid containing only the smaller (1.9×10^6 dalton) fragment complements *aroD6* mutant strains of *E. coli*.

The different levels of complementation obtained with pVK55 in various *aroD6* strains of *E. coli* point out a potential difficulty in attempting to clone eukaryotic functions in *E. coli*. Strain SK1485 apparently contained a mutation in addition to

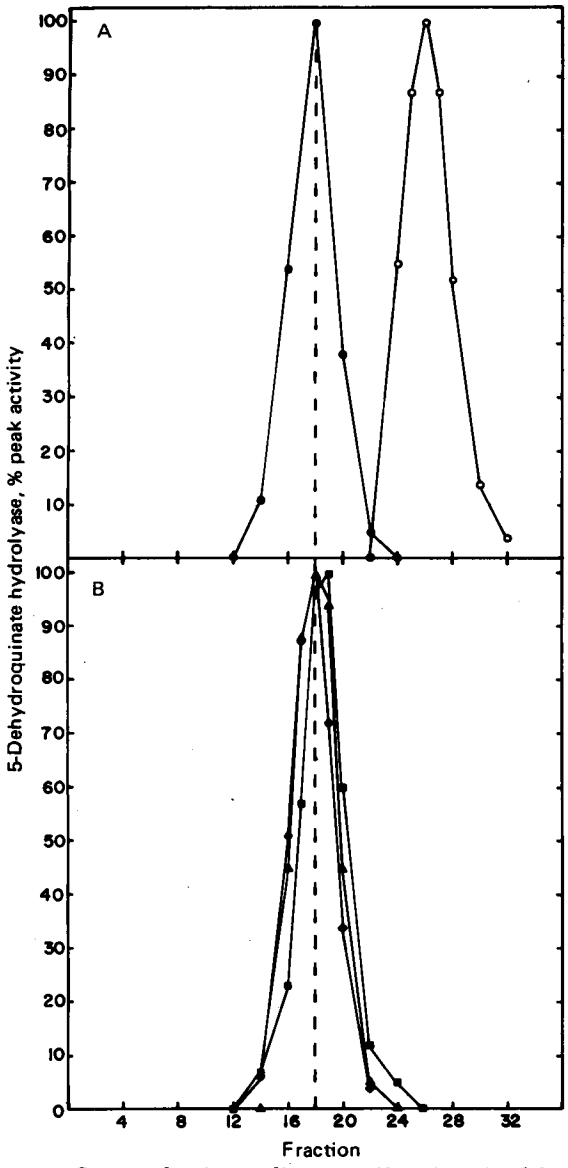

FIG. 3. Sucrose density gradient centrifugation of 5-dehydroquinate hydrolyase activity. (*A*) *N. crassa* strain M16 (●) and wild-type *E. coli* strain SK1313 (○). (*B*) pVK55 transformed strains SK1518 (■), SK1524 (♦), and SK1529 (▲).

aroD6 that prevented high levels of complementation and was only detected in the presence of the recombinant plasmid. This additional mutation was not present in AB1360, SK274, or SK1478 and was apparently introduced when SK1478 was made Str^R by ethyl methanesulfonate mutagenesis. In the absence of the spontaneous reversion or suppression of this allele in SK1518, pVK55 would not have been isolated. Therefore, care must be exercised in determining the genotype of strains to be used.

Another significant factor involved in the successful functional expression of the *N. crassa qa-2*$^+$ gene may be the unusual stability of catabolic dehydroquinase (9). In a prokaryotic background, eukaryotic enzymes with less inherent stability may be subject to proteolytic degradation or other conditions that prevent their detection.

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